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<b>(54) Title:</b> TARGETING AND DELIVERY OF GENES AND ANTIVIRAL AGENTS INTO CELLS BY THE ADENOVIRUS PENTON		
<b>(57) Abstract</b>  The present invention relates to gene therapy. In particular, therapeutic agents and methods useful in targeting and delivering non-native nucleotide sequences to specific cells are disclosed, wherein adenovirus penton, penton complex, or penton and fiber are used to facilitate targeting and delivery.		

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TARGETING AND DELIVERY OF GENES  
AND ANTIVIRAL AGENTS INTO CELLS  
BY THE ADENOVIRUS PENTON

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TECHNICAL FIELD

The present invention relates to gene therapy. In particular, therapeutic agents and methods useful in targeting and delivering genes to particular cells are disclosed, wherein adenovirus penton (a complex of penton base and fiber) or penton base is used to facilitate targeting and delivery.

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BACKGROUND

The primary impediment to the transfer of non-native or foreign DNA into mammalian cells is that the genetic material must be transported across multiple cellular barriers before it enters the host cell nucleus and initiates gene expression. Previously established methods have utilized artificial means to introduce DNA into the cell although these methods are associated with significant cell toxicity (Graham, et al., Virology 52: 456-467 (1973); Felgner, et al, PNAS USA 84: 7413-7417 (1987)).

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This problem has been partially overcome by using receptor-mediated endocytosis to deliver DNA into the cell. In this procedure, a molecular conjugate consisting of a ligand for a cell surface receptor (i.e. transferrin) is covalently linked to a DNA binding moiety (i.e., poly L-lysine) (Wu, et al., J. Biol. Chem. 262: 4429-4432 (1987); Wagner, et al., PNAS USA 87: 3410-3414 (1990); and Wagner, et al., PNAS USA 88: 4255-4259 (1991)). The conjugate is thus able to bind to the cell receptor and is subsequently internalized by endocytosis. In many cases, however, this approach is inefficient due to the targeting of the DNA conjugate to lysosomes (Zenke, et al., PNAS

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USA 87: 3655-3659 (1990); Cotten, et al., PNAS USA 87: 4033-4037 (1990)). Although lysosomatropic agents can sometimes be successfully used to inhibit this process, a high degree of variability is often  
5 observed in different cell types. (See, e.g., Curiel, et al., PNAS USA 88: 8850-8854 (1991).)

More recently, enhanced transfer of DNA conjugates into cells has been achieved with adenovirus, a human DNA virus which readily infects  
10 epithelial cells (Horwitz, "Adenoviridae and their replication", in Virology, Fields and Knipe, eds., Raven Press, NY (1990) pp. 1679-1740). Since adenovirus efficiently disrupts the membranes of  
15 endocytic vesicles, co-internalization of the virus with the DNA conjugate allows rapid transfer of the conjugate into the cell cytoplasm before it can be subjected to lysosomal degradation. The fact that adenovirus exhibits selective tropism for epithelial  
20 cells *in vivo* with the human cystic fibrosis transmembrane conductance regulator (CFTR) (Rosenfeld, et al., Cell 68: 143-155 (1992)) and the alpha 1-antitrypsin genes (Rosenfeld, et al., Science 252: 431-434 (1991)). Adenovirus type 2 has also been  
25 observed to facilitate the transfer of growth factors or toxins from endosomes into the cytoplasm of epithelial cells. (See, e.g., Fitzgerald, et al., Cell 32: 607-617 (1983); and Seth, et al., Mol. and Cell. Biol. 4: 1528-1533 (1984).)

30 Other advantages of adenovirus-mediated gene therapy include the efficient targeting of genes to epithelial cells, and the efficient delivery of genes to the cytoplasm, thus reducing lysosomal degradation of DNA. Further, the use of replication-defective  
35 strains of adenovirus reduces the cytopathic effect of

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replicating virus. Use of adenovirus may also facilitate the transfer of DNA into host cells. In vitro, adenovirus augments the delivery of transferrin-poly L-lysine/DNA conjugates into host cells. (See, e.g. Curiel, et al., PNAS USA 88: 8850-8854 (1991).) In vivo, alpha 1-antitrypsin and cystic fibrosis transmembrane conductance regulator protein genes have been delivered into epithelial cells using adenovirus type 5 (Rosenfeld, et al., Science 252: 431-434 (1991); Rosenfeld, et al., Cell 68: 143-155 (1992)).

#### BRIEF SUMMARY OF THE INVENTION

It has now been discovered that recombinant adenovirus penton or penton base, independent of the rest of the adenovirus genome, facilitates the transfer of exogenous or non-native genes into recipient cells. There are numerous advantages to the use of adenovirus penton or penton base for gene therapy over intact adenovirus or replication-deficient adenovirus, particularly in humans. For example, the adenovirus penton complex likely possesses all of the functional properties required for gene therapy including binding to epithelial cell receptors, stimulation of endocytosis, and penetration of endocytic vesicles. In addition, large amounts of recombinant penton base and fiber may be produced in insect cells via the use of an expression system such as the baculovirus expression system. The proteins so produced are capable of assembling into the penton complex.

Chimeric forms of recombinant penton complex may also be constructed with the capacity to target foreign genes to non-epithelial cells. These chimeric forms are also capable of evading recognition and attack by the host's immune system. Furthermore, the

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use of penton complex and fiber is a much safer alternative for gene therapy applications than the introduction of adenovirus genes into mammalian cells as some forms of adenovirus contain genes that can transform cells. Removal of those deleterious genes from an adenovirus vector may not in itself be enough to prevent transformation as the vector may combine with a natural virus to produce a more pathogenic strain. The use of recombinant or conventionally purified penton base or penton complex of adenovirus overcomes these secondary *in vivo* events.

Therefore, in one aspect, the present invention contemplates a therapeutic agent capable of specifically targeting epithelial cells comprising an adenovirus penton base operatively linked to a nucleotide sequence encoding a mammalian polypeptide and an active promoter. In one embodiment, the nucleotide sequence is conjugated directly to a region of the penton which does not alter its functional properties.

In various alternative embodiments, the nucleotide sequence may be "sense" or "antisense". In another embodiment, the penton base or penton complex is incorporated into liposomes which contain the foreign therapeutic nucleotide sequence or antisense oligonucleotides. The invention also contemplates the penton-mediated delivery of plasmid-based vectors containing the gene of interest under the control of strong constitutive or inducible promoters and/or enhancer elements to obtain higher levels of gene expression than that obtained by incorporating the target gene into the intact adenovirus genome.

Thus, in one embodiment, the present invention contemplates a composition designed to specifically target epithelial cells and deliver a therapeutic

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nucleotide sequence to the cells, comprising an adenovirus-derived protein and the nucleotide sequence. In one variation, the protein is selected from the group consisting of penton base and penton complex. In the various embodiments contemplated herein, the protein may be conventionally-purified or recombinant, and may further be derived from adenovirus type 2. The present invention further contemplates that a composition may further comprise a pharmaceutically acceptable carrier or excipient.

In alternative embodiments, the adenovirus-derived protein may be a complete protein, an apoprotein, a polypeptide, or an amino acid residue sequence comprising three or more amino acid residues linked together via peptide linkages. In all embodiments, the adenovirus-derived protein is biologically active, and is preferably non-toxic.

In another embodiment of the invention, the therapeutic nucleotide sequence is operatively linked to the adenovirus-derived protein. In another variation, the therapeutic nucleotide sequence and the adenovirus-derived protein are contained within a liposome. In yet another embodiment, the nucleotide sequence encodes a polypeptide and further comprises an active promoter for expressing the polypeptide. In variations thereof, the promoter is selected from the group consisting of constitutive and inducible promoters. In one variation, the nucleotide sequence is a "sense" sequence; in another, the nucleotide sequence is an "antisense" sequence.

The present invention further contemplates the use of a composition comprising an adenovirus-derived protein and a therapeutic nucleotide sequence in the manufacture of a medicament for specifically targeting epithelial cells and delivering an effective amount of

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the therapeutic nucleotide sequence to the cells.

In another variation, the invention contemplates the use of a compound comprising a therapeutic nucleotide sequence operatively linked to an  
5 adenovirus-derived protein in the manufacture of a medicament for specifically targeting epithelial cells and delivering an effective amount of the therapeutic nucleotide sequence to the cells. In one embodiment, the adenovirus-derived protein is selected from the  
10 group consisting of penton base and penton complex. In other variations, it will be appreciated that any combination of the preceding elements may also be efficacious for the uses as described herein.

The invention further contemplates methods of  
15 making a medicament useful in specifically targeting and delivering a therapeutic nucleotide sequence to mammalian cells, comprising operatively linking an adenovirus-derived amino acid residue sequence and the nucleotide sequence and incorporating the operatively  
20 linked sequences into a liposome. In one variation, the amino acid residue sequence comprises penton base or penton complex. Other variations include combinations of elements as described hereinabove.

The present invention further contemplates an  
25 article of manufacture comprising packaging material and a composition effective for targeting epithelial cells and delivering a therapeutic nucleotide sequence to the cells, wherein the composition comprises an adenovirus-derived protein and a therapeutic  
30 nucleotide sequence, and wherein the packaging material comprises a label which indicates that the composition can be used for targeting epithelial cells and delivering a therapeutic nucleotide sequence to the cells.

35 In another embodiment, the invention contemplates



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a composition useful in specifically targeting non-epithelial cells and delivering a therapeutic nucleotide sequence to the cells, comprising an adenovirus-derived protein-ligand conjugate and the nucleotide sequence.

In yet another embodiment, the invention contemplates a composition designed to specifically target epithelial cells and deliver a therapeutic nucleotide sequence to the cells, comprising a protein or polypeptide including an RGD amino acid residue sequence, and the nucleotide sequence. In one variation, the composition is designed to specifically target endothelial cells. In yet another variation, the composition is designed to target malignant or tumor cells.

In various embodiments, the adenovirus-derived protein is selected from the group consisting of penton base and penton complex. In another embodiment, the composition is immunotherapeutic. In one variation, the composition is designed to target cells expressing integrins, including, in various embodiments, receptors for vitronectin, fibronectin, collagen, laminin, thrombospondin, fibrinogen, and von Willebrandt's factor. In one variation, the composition is designed to specifically target cells expressing a vitronectin receptor. In other variations, the composition is designed to specifically target cells expressing an  $\alpha_v\beta_3$  or an  $\alpha_v\beta_5$  receptor.

In various embodiments of the invention, the therapeutic nucleotide sequence is operatively linked to the adenovirus-derived protein. In another variation, the therapeutic nucleotide sequence and the adenovirus-derived protein are contained within a

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liposome.

In various embodiments, the nucleotide sequence encodes a polypeptide and further comprises an active promoter for expressing the polypeptide. In one  
5 variation, the promoter is selected from the group consisting of constitutive and inducible promoters.

In alternative embodiments of the invention, the ligand may comprise an antibody, or may comprise an attachment sequence for a receptor.

10 The present invention further contemplates a method of specifically targeting and delivering a therapeutic nucleotide sequence into mammalian cells, comprising administration of a composition comprising an adenovirus-derived protein and the nucleotide  
15 sequence. In some variations, the adenovirus-derived protein is selected from the group consisting of penton base and penton complex. In various embodiments, the adenovirus-derived protein is recombinant; in others, the adenovirus-derived protein  
20 is conventionally-purified. The invention further contemplates that the adenovirus-derived protein is derived from adenovirus type 2.

In alternative variations of the invention contemplated herein, the therapeutic nucleotide  
25 sequence is operatively linked to the adenovirus-derived protein. In other embodiments, the therapeutic nucleotide sequence and the adenovirus-derived protein are contained within a liposome.

The invention further contemplates that compounds  
30 and compositions of the present invention may be administered in various forms and by various means, including administration as an aerosol spray, intraperitoneally, via surgical implantation or injection, or via perfusion.

35 In various embodiments, a composition according

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to the present invention is administered in a dosage range of 1  $\mu$ g/ml to 1 mg/ml of active ingredient.

It will be appreciated that any combination of the preceding elements may also be efficacious as described herein.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a schematic representation of an adenovirus. The mobilities and relative amounts of each protein after electrophoresis of the dissociated virus on a sodium dodecyl sulfate-containing polyacrylamide gel are shown on the right. The position of each polypeptide in the virion is designated; however, the configuration of the DNA does not imply the actual structure within the core. The Roman numerals refer to a polypeptide designation described by Maizel, et al. (Virology 36: 126-136 (1968)). The hexon (II), penton base (III), fiber (IV), and hexon-associated proteins (IIIa, VI, VIII, and IX) are subunits of the capsid. The core contains proteins V, VII, and u, as well as the 55 kD terminal protein covalently linked at each of the 5' ends of the linear DNA. The two molecules of TP per virion are too few to be demonstrated by the Coomassie stain of the viral polypeptides, and u probably is one of the polypeptides found in the region of the gel containing proteins X to XII. (See Persson, et al., Curr. Top. Microbiol. Immunol. 97: 157-203 (1982).)

Figure 2 (A and B) illustrates a diagram of the adenovirus particle. Fig. 2A is a diagram illustrating the adenovirus particle, showing the location of major protein sub-assemblies. The nine hexons drawn as a group and the "peripentonal" hexons are trimers of the same polypeptide. They are distinguished only by their location in the structure. (See Burnett, in McPherson, et al. (eds.), Biological

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Macromolecules and Assemblies, Vol. I, "The Viruses", Wiley, NY, pp. 337-385 (1984).) Fig. 2B illustrates a cross-section, and shows the probable location of the principal polypeptide components and the viral DNA.

5 The assignment for proteins other than II (hexon), III (penton base), and IV (penton fiber) is based largely on studies of stepwise dissociation. (See Ginsberg, in Fraenkel-Conrat, et al. (eds.), Comprehensive Virology, Vol. 13, Plenum, NY, pp. 409-457 (1979).)

10 Figure 3 illustrates DNA delivery into cells in culture via the recombinant penton base of adenovirus. (Refer to Example 3 for details of the gene transfer experiment.) Sample numbers are listed on the horizontal axis, whereas luciferase activity (in light  
15 units) is plotted on the vertical axis. The abbreviation "PL" refers to poly L-lysine and pRSVLuc is a reporter plasmid containing a luciferase gene. The samples identified by numbers 1-5 contained the following: (1) transferrin/PL, but no pRSVLuc; (2)  
20 pRSVLuc + transferrin/PL; (3) pRSVLuc + transferrin/PL + penton base + fiber; (4) pRSVLuc + transferrin/PL + penton base; (5) pRSVLuc + transferrin/PL + fiber.

#### DETAILED DESCRIPTION

##### A. Definitions

25 Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can  
30 be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH<sub>2</sub> refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the

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carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 C.F.R. §1.822(b)(2)), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE			
SYMBOL		AMINO ACID	
	<u>1-Letter</u>	<u>3-Letter</u>	
10	Y	Tyr	tyrosine
	G	Gly	glycine
	F	Phe	phenylalanine
	M	Met	methionine
15	A	Ala	alanine
	S	Ser	serine
	I	Ile	isoleucine
	L	Leu	leucine
	T	Thr	threonine
20	V	Val	valine
	P	Pro	proline
	K	Lys	lysine
	H	His	histidine
	Q	Gln	glutamine
25	E	Glu	glutamic acid
	Z	Glx	Glu and/or Gln
	W	Trp	tryptophan
	R	Arg	arginine
	D	Asp	aspartic acid
30	N	Asn	asparagine
	B	Asx	Asn and/or Asp
	C	Cys	cysteine
	X	Xaa	Unknown or other

35 It should be noted that all amino acid residue

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sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxy-terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids, such as those listed in 37 C.F.R. §1.822(b)(4), and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or to an amino-terminal group such as  $\text{NH}_2$  or to a carboxy-terminal group such as  $\text{COOH}$ .

DNA Homolog: A nucleic acid having a preselected conserved nucleotide sequence and a sequence encoding a preferred polypeptide according to the present invention.

Downstream: Further along a DNA sequence in the direction of sequence transcription or read out, that is traveling in a 3'- to 5'-direction along the non-coding strand of the DNA or 5'- to 3'-direction along the RNA transcript.

Expression: The process undergone by a structural gene to produce a polypeptide. It is a combination of transcription and translation.

Gene: A nucleic acid whose nucleotide sequence encodes an RNA or polypeptide. A gene can be either RNA or DNA. Genes may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Hybridization: The pairing of substantially complementary nucleotide sequences (strands of nucleic acid) to form a duplex or heteroduplex by the

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establishment of hydrogen bonds between complementary base pairs. It is a specific, i.e. non-random, interaction between two complementary polynucleotides that can be competitively inhibited.

5        Ligand: A molecule that contains a structural portion that is bound by specific interaction with a particular receptor protein.

10        Nucleotide: A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate group, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. When the  
15        nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose, it is referred to as a nucleotide. A sequence of operatively linked nucleotides is typically referred to herein as a "base sequence" or "nucleotide sequence", and their grammatical equivalents, and is represented herein by  
20        a formula whose left to right orientation is in the conventional direction of 5'-terminus to 3'-terminus.

25        Nucleotide Analog: A purine or pyrimidine nucleotide that differs structurally from A, T, G, C, or U, but is sufficiently similar to substitute for the normal nucleotide in a nucleic acid molecule.

30        Oligonucleotide or Polynucleotide: A polymer of single or double stranded nucleotides. As used herein, "oligonucleotide" and its grammatical equivalents will include the full range of nucleic acids. An oligonucleotide will typically refer to a nucleic acid molecule comprised of a linear strand of ribonucleotides. The exact size will depend on many factors, which in turn depends on the ultimate conditions of use, as is well known in the art.

35        Penton: The terms "penton" or "penton complex"

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are preferentially used herein to designate a complex of penton base and fiber. The term "penton" may also be used to indicate penton base, as well as penton complex. The meaning of the term "penton" alone should be clear from the context within which it is used.

Plasmid: An autonomous self-replicating extrachromosomal circular DNA.

Polypeptide and Peptide: These terms are used interchangeably herein to designate a series of no more than about 50 amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

Protein: As used herein, this term designates a linear series of greater than 50 amino acid residues connected one to the other as in a polypeptide.

Receptor: Receptor and receptor protein are terms used herein to indicate a biologically active proteinaceous molecule that specifically binds to (or with) other molecules.

Recombinant DNA (rDNA) molecule: A DNA molecule produced by operatively linking a nucleic acid sequence, such as a gene, to a DNA molecule sequence of the present invention. Thus, a recombinant DNA molecule may be a hybrid DNA molecule comprising at least two nucleotide sequences not normally found together in nature. rDNAs not having a common biological origin, i.e., evolutionarily different, are said to be "heterologous".

Therapeutic Nucleotide Sequence: As described and claimed herein, such a sequence includes DNA and RNA sequences encoding an RNA or polypeptide. Such sequences may be "native" or naturally-derived sequences; they may also be recombinantly-derived sequences. Therapeutic nucleotide sequences therefore



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include antisense sequences or nucleotide sequences which may be transcribed into antisense sequences. Therapeutic nucleotide sequences further comprise sequences which function to produce a desired effect in the cell or cell nucleus into which said therapeutic sequences are delivered. For example, a therapeutic nucleotide sequence may encode a functional protein intended for delivery into a cell which is unable to produce that functional protein.

5  
10     Transfection: The acquisition of new genetic markers by incorporation of added DNA in eucaryotic cells.

15     Transformation: The acquisition of new genetic markers by incorporation of added DNA in procaryotic cells.

20     Upstream: In the direction opposite to the direction of DNA transcription, and therefore going from 5' to 3' on the non-coding strand, or 3' to 5' on the mRNA.

25     Vector: A polynucleotide molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment.

30     Cloning Vector: Any plasmid or virus into which a foreign DNA may be inserted to be cloned.

35     Expression Vector: Any plasmid or virus into which a foreign DNA may be inserted or expressed. Vectors capable of directing the expression of DNA segments (genes) encoding one or more proteins are referred to herein as "expression vectors". Also included are vectors which allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

40     Leader or Signal Polypeptide: A short length of

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amino acid sequence at the amino end of a protein, which carries or directs the protein through the inner membrane and so ensures its eventual secretion into the periplasmic space. The leader sequence peptide is commonly removed before the protein becomes active.

Reading Frame: A particular sequence of contiguous nucleotide triplets (codons) employed in translation. The reading frame depends on the location of the translation initiation codon.

10 B. Adenoviruses

Since its discovery in 1953, adenovirus has served as a model for molecular biology and cell transformation. The pentagonal capsomer (the penton) at the vertex of the adenovirus icosahedron consists of a fiber projection, linked by noncovalent bonds to the penton base, anchored in the capsid. (See, e.g., Novelli and Boulanger, Virology 185: 365-376 (1991); Nermut, in The Adenoviruses, Ginsberg, ed., Plenum, NY, pp. 5-34 (1984); and Pettersson, in The Adenoviruses, Ginsberg, ed., Plenum, NY, pp. 205-207 (1984).)

The adenovirus (Ad) particle is relatively complex and may be resolved into various substructures. The outer shell is strikingly icosahedral in shape and, at first glance, appears to have a triangulation number of 25. The structures at the fivefold positions ("pentons") are different from the rest ("hexons"), however, and the hexons are chemically trimers rather than hexamers. Thus, the structure really does not correspond to a simple sub-triangulated icosahedral design. (See, e.g., Fields, et al., Virology, Vol. I, Raven Press, NY, pp. 54-56 (1990).)

Adenoviruses are nonenveloped, regular icosahedrons (20 triangular surfaces and 12 vertices)

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that are about 65-80 nm in diameter (about 1400 angstroms (Å)). A structure, called fiber, projects from each of the vertices. The length of the fiber varies with the adenovirus serotype. The protein coat or capsid is composed of 252 subunits (capsomeres), of which 240 are hexons and 12 are pentons. Each of the pentons contains a penton base on the surface of the capsid and a fiber projecting from the base, which is surrounded by five hexons. The name "penton" is derived from these geometric relationships. All the other capsomeres are hexons, and they are so named because each is surrounded by six neighboring identical structures. A group of nine hexons (the "ninemers") can be purified from each of the 20 triangular faces of the virion by gentle lysis of the particles with 10% pyridine. The hexons and pentons are each derived from different viral polypeptides. Recently, combined electron-microscopic and crystallographic approaches have produced detailed descriptions of hexon-hexon and hexon-penton base interactions that have helped clarify the relationship of the virus subunits. In addition, the hexon capsomere was shown to contain two  $\beta$  barrel configurations that were very similar to structures contained on the surface of several picornaviruses (poliovirus and rhinovirus). Disruption of the virion either with 5M urea, 10% pyridine, acetone, or multiple freeze-thaw cycles results in a core structure composed of the double-strand DNA with four additional viral proteins not appearing in the capsid. Adenoviruses contain 13% DNA and 87% protein, have no membranes or lipids, and are therefore stable in solvents such as ether and ethanol. The virion contains fiber polypeptides modified by covalent addition of glucosamine and has a density of 1.34

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g/cm<sup>3</sup> in CsCl.

With regard to virion capsid polypeptides, most of the detailed structural studies of adenovirus polypeptides have been performed for Ad types 2 and 5.

5 The following discussion emphasizes Ad 2 data.

Variation in molecular weights of the analogous proteins among the various serotypes is small but has been useful in one of the adenovirus classification schemes. In Fig. 1, the adenovirus polypeptides, as well as their positions in the virion, are illustrated. There is no polypeptide I because the originally designated protein I was a mixture of smaller molecules that formerly were difficult to dissociate. Polypeptide II is the 120 kilodalton (kd) hexon.

15 The hexon capsomere is a trimer of three identical 110K polypeptides, conventionally denoted polypeptide II, held tightly together by noncovalent interactions. The 240 hexon capsomeres in each virion therefore contain 720 identical polypeptides. The high-resolution structure shows that each subunit contains two rather similar  $\beta$ -barrel domains, in which the course of the polypeptide chain is somewhat like that in an RVC (RNA virus capsid) fold. That is, in the hexon domains there are two sheets of four strands each, connected sequentially as in the framework of an RVC domain. However, the shape of the domain is rather different; the strands run radially rather than tangentially to the shell, and the major loops are between strands D and E and strands F and G. Thus, use of a similarly folded design may not imply any evolutionary or functional relationship. The DE and FG loops from both domains project outward, interacting tightly with corresponding loops from the

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other two subunits in the trimer. The conformation of these loops therefore depends on trimer formation.

The loops bear the principal type-specific antigenic determinants. Assembly of the hexon from newly synthesized protein in vivo appears to require a factor, the "100K protein", which is also encoded by the virus. This protein does not form part of the final structure. Dissociation of adenovirus particles by various methods yields groups of nine hexons, as shown in Fig. 2A. They are derived from virions as indicated, and they include all but the peripentonal hexons. The groups of nine are held together by viral protein IX (see Fig. 2B), which copurifies with these structures. Its location has been determined by scanning electron microscopy. (See, e.g., Fields, et al., Virology, Vol. I, Raven Press, NY, pp. 54-56 (1990).)

Polypeptides VI (24 kd), VIII (13 kd), and IX (12 kd) are associated with the hexon after various isolation procedures. For example, after disruption of the virion with 10% pyridine, polypeptides VI and IX are isolated together with hexon ninemers. Polypeptides VI and VIII are synthesized as larger precursors and are cleaved during assembly. The positions of these proteins within the virion are shown in Fig. 1, and those polypeptides facing the outer surface of the particle are indicated. The location of polypeptides was obtained by surface labeling of virion with iodinated lactoperoxidase or by analysis of various degradation products of gently disrupted particles. The penton base (polypeptide III, 85 kd) is noncovalently attached to fiber (polypeptide IV, 62 kd).

The adenovirus penton is a noncovalently associated complex of two proteins, the fiber and

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penton base. In general, the penton complex comprises three fiber monomers associated with one penton base. (See, e.g., van Oostrum and Burnett, J. Virol. 56: 439-448 (1985).) The complex of penton base plus  
5 fiber polypeptides is called penton capsomere. There are five molecules of polypeptide III per penton base, as well as three molecules of polypeptide IV in the fiber at each vertex. The outer tip of the penton  
10 fiber is thought to be the site of attachment to cellular receptors. The orientation of the fiber polypeptide has been determined. The conserved amino terminus is attached to the penton base, whereas the knob containing the carboxy end projects farthest from the capsid. There is a conserved structural motif in  
15 the shaft of the fiber which contains periodic repeats of prolines and hydrophobic residues. The amino acid sequence of the shaft, however, is not well conserved, which correlates with the antigenic variation of fiber characteristic of the different virus types.  
20 Polypeptide IIIa (66 kd) is associated with the peripentonal hexons after pyridine dissociation of virions. (See, e.g., Fields, et al., Virology, Vol. II, Raven Press, NY, pp. 1681-1682 (1990).)

The fiber is an elongated protein (180,000 kDa)  
25 which exists as a trimer of three identical polypeptides of 582 amino acids in length (62 kDa). The N-terminus of the fiber mediates binding to the penton base while the C-terminus is involved in initial binding of the virus to cellular receptors.  
30 Cellular receptors for the fiber have not yet been identified. Adenovirus attachment to a putative host cell receptor is mediated by the fiber protein as demonstrated by the ability of soluble fiber to completely block virus attachment and infection. The  
35 elongated portion of the fiber (shaft) is comprised of

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22 repeats of 15 amino acids each (adenovirus type 2) which form amphipathic B sheet structure.

5 The adenovirus penton base is a pentamer comprised of 5 identical subunits of 571 amino acids which are noncovalently associated to form a ring-shaped complex of approximately 200 X 90 Å. There is a 98% sequence identity in the penton base within the same subgroup of adenoviruses while there is only 69% identity in the fiber. The penton base  
10 contains an arginine-glycine-aspartic acid-containing (RGD-containing) peptide sequence (i.e., HAIRGDTFA (SEQ ID NO 1), residues 340-342 within residues 337-345) which are now shown to be involved in the attachment of the virus to integrin receptors. Such  
15 binding is required for internalization of adenovirus into the host cell. Synthetic peptides containing the RGD sequence inhibit virus infection as well as virus entry into the host cell. Thus, adenovirus, which is a nonenveloped DNA virus, utilizes separate proteins for  
20 attachment and entry in a manner similar to enveloped human viruses.

Adenoviruses attach to host-cell receptors via the penton fiber glycoprotein and enter cells through the process of receptor-mediated endocytosis mediated  
25 by the penton base. The penton fiber projects from the vertices of the virion capsid. It has a small distal knob that contains the cell attachment site. It has been estimated that there are  $10^4$  virion binding sites per cell. There are a significantly  
30 larger number of cellular binding sites for the isolated penton fiber protein, indicating that the intact virion occupies several receptor sites. Antibodies directed against the penton fiber neutralize viral infectivity. Affinity columns made

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with the adenovirus penton capsomer have been used to isolate and partially purify the receptor for adenovirus on KB cells. Three proteins of molecular mass 34, 42, and 78 kd have been isolated using this technique. These proteins have been presumed to comprise different forms of the same molecule or polypeptides which together form a part of a receptor complex.

The 35,000 base pair (bp) genome of adenovirus type 2 has been sequenced and the predicted amino acid sequences of the major coat proteins (hexon, fiber and penton base) have been described. (See, e.g., Neumann et al., Gene 69:153-157 (1988); Herisse et al., Nuc. Acids Res. 9:4023-4041 (1981); Roberts et al., J. Biol. Chem. 259:13968-13975 (1984); Kinloch et al., J. Biol. Chem. 259:6431-6436 (1984); and Chroboczek et al., Virology 161:549-554 (1987).) The core of adenovirions contains DNA (about 30 kb) and two basic proteins (V and VII). The arginine-rich protein VII is present in about 1070 copies/particle, and it can neutralize about 50% of the DNA phosphates. Isolated cores are compact particles, but without any very striking substructures.

Adenovirus DNA is about  $23.85 \times 10^6$  daltons for adenovirus type 2 (Ad 2) and varies slightly in size, depending on serotype. Ad DNA is approximately 11 micromolar ( $\mu\text{m}$ ) in length and has the unusual feature of a virus-encoded 55 kd terminal polypeptide (TP) covalently linked to dCMP at each 5' end of the linear genome. Extensive proteolysis does not remove all the amino acids from the 5' terminal dCMP; however, the protein-DNA bond involving a serine hydroxyl group is alkaline-labile in sodium hydroxide or piperidine. Because TP does not allow the covalently linked



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fragments of DNA to enter agarose gels during electrophoresis without detergents, the end fragments of DNA can easily be determined for new serotypes.

5 Via electron microscopy, approximately half the Ad DNA molecules released from virions with 4M guanidine can be seen as circles formed by noncovalent interactions between the terminal proteins at each end. Adenovirus DNA has inverted terminal  
10 redundancies and the 100- to 140-base-pair length of these repeats varies with the serotype. Therefore, denatured single strands of adenovirus DNA can form circles by base pairing at the ends of the DNA, and these "panhandle" structures may be important in DNA replication.

15 Extensive restriction endonuclease maps exist for many Ad serotypes. The base compositions of some Ad serotypes vary from 48% to 61% G+C content, and this is one criterion for classifying the human adenoviruses. Considerable DNA sequencing data are  
20 currently available, especially for Ad serotypes 2, 5, 7, and 12 (Baum, et al., J. Virol. 10: 211-219 (1972); Tooze, DNA Tumor Viruses: Molecular Biology of Tumor Viruses, 2d ed., Cold Spring Harbor Laboratory, NY, pp. 937-1036 (1981); van Ormondt, et al., Curr. Top. Microbiol. Immunol. 110: 73-142 (1984)).  
25

Although adenovirus-mediated gene therapy represents an improved method of DNA transfer into cells, a potential limitation of this approach is that adenovirus replication results in disruption of the  
30 host cell. In addition, adenovirus also possesses oncogenic properties including the ability of one of its proteins to bind to tumor suppressor gene products. The use of replication defective strains of adenovirus which render the virus unable to replicate  
35 in host cells is in principle more suitable for in

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vivo therapy; however, the potential of co-infection of epithelial cells with wild-type strains of virus resulting in transactivation of the recombinant virus may represent a significant safety concern for in vivo applications. Furthermore, it is not yet known whether recombinant adenoviruses are capable of integrating their genome into host cell DNA allowing for long-term stable expression of the foreign gene.

Another undesirable aspect of using intact or replication-deficient adenovirus as a gene transfer means is that it is an oncogenic virus whose gene products are known to interfere with the function of host cell tumor suppressor proteins as well as immune recognition molecules, such as the major histocompatibility complex (MHC). In addition, pre-existing circulating antibodies to adenovirus may significantly reduce the efficiency of in vivo gene delivery. Lastly, only a foreign gene of 6 kilobases (kb) or less can be incorporated into the intact adenovirus genome for gene transfer experiments while DNA segments of greater than 15 kb can be transferred using the methods of this invention.

The present invention uses a coat protein subunit of adenovirus known as the penton which duplicates cell receptor binding and DNA delivery properties of intact adenovirus virions and thus represents an improved method for gene therapy as well as antisense-based antiviral therapy. The penton is composed of a fiber protein which mediates receptor binding and the penton base (Boudin, et al., Virology 116: 589-604 (1982)) which mediates various entry and also disruption of the endocytic vesicle following exposure to a low pH environment thus allowing virus entry into the cytoplasm (Seth, et al., J. Biol. Chem. 260: 9598-9602 (1985); Seth, et al., J. Biol. Chem.

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259: 14350-14353 (1984)).

In contrast to the disadvantages of using intact or replication-deficient adenovirus, the use of adenovirus-derived penton base and fiber proteins provides certain advantages for gene delivery. The adenovirus penton complex likely possesses all of the functional properties required for gene therapy including binding to epithelial cell receptors and penetration of endocytic vesicles. Second, while penton base and fiber proteins can be purified directly from adenovirus using conventional purification techniques as described in Example 2, large amounts of recombinant penton base and fiber can be produced in insect cells using baculovirus as well as from other various expression systems following amplification of genes encoding the penton base and fiber. While the adenovirus type 2-derived recombinant penton base and fiber are preferred for use in this invention, the fiber and penton base proteins purified by conventional methods from adenovirus as described in Example 2 are also contemplated for use in this invention.

The recombinant proteins can be amplified from the available human adenovirus serotypes from type 1 through 47 currently available from American Type Culture Collection (ATCC), Rockville, MD. Both recombinant and conventionally purified penton base and fiber proteins are capable of assembling into the penton complex. Use of adenovirus penton also eliminates the safety concerns related to the introduction of intact adenovirus genes into human cells.

Recombinant or conventionally purified adenovirus type 2 penton as well as the noncomplexed penton base and fiber proteins used independently are used in this

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invention to facilitate specific targeting and delivery of foreign genes into epithelial cells. For this purpose, DNA or antisense-based plasmids are conjugated directly to a region of the penton such that its functional properties are not altered. Alternatively, the penton complex, penton base or fiber is incorporated into liposomes which contain the foreign gene or antisense oligonucleotides. The invention also uses plasmid-based vectors containing the gene of interest under the control of strong constitutive or inducible promoters and/or enhancer elements as described in Example 5 to obtain higher levels of gene expression than that obtained by incorporating the target gene into the adenovirus genome.

An extension of this invention is that the adenovirus penton will be used to target and deliver genes into non-epithelial cells by incorporating the attachment sequence for other receptors such as CD4 on T cells onto the fiber protein by recombinant DNA techniques, thus producing a chimeric molecule as described in Example 5. This should result in the ability to target and deliver genes into a wide range of cell types with the advantage of evading recognition by the host's immune system. The penton-based gene delivery system thus provides for increased flexibility in gene design to enable stable integration into proliferating and nonproliferating cell types. For example, this is contemplated by the incorporation of a retrovirus genome into the penton-based gene delivery system, the result of which allows for the stable integration of the exogenous gene into the recipient cell mediated by the retrovirus while allowing for delivery into nonproliferating cells mediated by the penton. Gene

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transfer mediated by retroviruses outside this context is limited to proliferating cell types. For a review of gene transfer by using retroviral vectors, see International Applications WO 92/07943 and WO 92/07959, the disclosures of which are hereby incorporated by reference. Thus, increased flexibility and enhanced stable gene transfer is attained by coupling the two gene transfer systems.

One application of this invention is gene therapy for common hereditary disorders involving respiratory epithelium such as alpha 1-antitrypsin deficiency resulting in emphysema and cystic fibrosis transmembrane conductance regulator (CFTR) deficiency resulting in cystic fibrosis. A second application is delivery of antiviral agents such as antisense compounds or ribozymes to epithelial cells as described in Example 5. The adenovirus penton may also be useful in the enhanced delivery of immunotoxins for the treatment of various cancers.

C. Expression and Purification of Recombinant Penton Base and Fiber

1. PCR Amplification of Penton Base and Fiber Proteins

Using PCR, it is possible to synthesize useful polypeptide-encoding nucleotide sequences which may then be operatively linked to a vector and used to transform an appropriate cell and expressed therein.

Particularly preferred methods for producing large quantities of recombinant penton base and fiber proteins of the present invention rely on the use of preselected oligonucleotides as primers in a polymerase chain reaction (PCR) to form PCR reaction products as described herein. PCR is also used in the preparation of the therapeutic compositions of this invention as described below. For this embodiment,

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preparation of genes or nucleotide sequences for therapeutic gene delivery is typically accomplished by primer extension, preferably by primer extension in a polymerase chain reaction (PCR) format.

5        If the DNA products described above are to be produced by (PCR) amplification, two primers, i.e., a PCR primer pair, must be used for each coding strand of nucleic acid to be amplified. The first primer becomes part of the nonsense (minus or complementary) strand and hybridizes to a nucleotide sequence  
10       conserved among the preferred gene's plus (or coding) strands. To produce coding DNA homologs, first primers are therefore chosen to hybridize to (i.e. be complementary to) conserved regions within the gene(s)  
15       of choice.

      Second primers become part of the coding (plus) strand and hybridize to a nucleotide sequence conserved among minus strands. To produce the coding DNA homologs, second primers are therefore chosen to  
20       hybridize with a conserved nucleotide sequence at the 5' end of the coding gene such as in that area coding for the leader or first framework region. It should be noted that in the amplification of the coding DNA homologs the conserved 5' nucleotide sequence of the  
25       second primer can be complementary to a sequence exogenously added using terminal deoxynucleotidyl transferase as described by Loh et al., Science 243: 217-220 (1989). One or both of the first and second primers can contain a nucleotide sequence defining an  
30       endonuclease recognition site (restriction site). The site can be heterologous to the gene being amplified and typically appears at or near the 5' end of the primer.

      The first primer of a PCR primer pair is  
35       sometimes referred to herein as the "sense primer"

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because it hybridizes to the coding or sense strand of a nucleic acid. In addition, the second primer of a PCR primer pair is sometimes referred to herein as the "antisense primer" because it hybridizes to a non-coding or antisense strand of a nucleic acid, i.e., a strand complementary to a coding strand. A plurality of first primers and/or a plurality of second primers can be used in each amplification, e.g., one species of first primer can be paired with a number of different second primers to form several different primer pairs. Alternatively, an individual pair of first and second primers can be used. Primers are also referred to as being either 5' or 3' primers indicating the ends or region of the DNA to which the primers hybridize. In this case, the 5' and 3' primers are respectively the antisense and sense primers.

When present, the restriction site-defining portion is typically located in a 5'-terminal non-priming portion of the primer. The restriction site defined by the first primer is typically chosen to be one recognized by a restriction enzyme that does not recognize the restriction site defined by the second primer, the objective being to produce a DNA molecule having cohesive termini that are non-complementary to each other and thus allow directional insertion into a vector.

In PCR, each primer works in combination with a second primer to amplify a target nucleic acid sequence. The choice of PCR primer pairs for use in PCR is governed by various considerations, as discussed herein. That is, the primers have a nucleotide sequence that is complementary to a sequence conserved in the gene of choice. Useful priming sequences are disclosed hereinafter.

The strategy used for cloning the selected genes

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will depend, as is well known in the art, on the type, complexity, and purity of the nucleic acids making up the various genes. Other factors include whether or not the genes are to be amplified and/or mutagenized.

5           In general, the exemplary genes are comprised of polynucleotide coding strands, such as mRNA and/or the sense strand of genomic DNA. If the polynucleotide sequence is in the form of double stranded genomic DNA, it is usually first denatured, typically by  
10           melting, into single strands. A gene sequence is subjected to a PCR reaction by treating (contacting) the sequence with a PCR primer pair, each member of the pair having a preselected nucleotide sequence. The PCR primer pair is capable of initiating primer  
15           extension reactions by hybridizing to nucleotide sequences, preferably at least about 10 nucleotides in length and more preferably at least about 20 nucleotides in length, conserved within the gene sequence.

20           The PCR reaction is performed by mixing the PCR primer pair, preferably a predetermined amount thereof, with the nucleic acids of the selected gene or DNA nucleotide sequence, preferably a predetermined amount thereof, in a PCR buffer to form a PCR reaction  
25           admixture. The admixture is maintained under polynucleotide synthesizing conditions for a time period, which is typically predetermined, sufficient for the formation of a PCR reaction product, thereby producing a plurality of different  
30           polypeptide-encoding DNA homologs.

          The PCR reaction is performed using any suitable method. Generally it occurs in a buffered aqueous solution, i.e., a PCR buffer, preferably at a pH of 7-9, most preferably about 8. Preferably, a molar



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excess (for genomic nucleic acid, usually about  $10^6$ :1 primer:template) of the primer is admixed to the buffer containing the template strand. A large molar excess is preferred to improve the efficiency of the process.

5 The PCR buffer also preferably contains the deoxyribonucleotide triphosphates dATP, dCTP, dGTP, and dTTP and a polymerase, typically thermostable, all in adequate amounts for primer extension

10 (polynucleotide synthesis) reaction. The resulting solution (PCR admixture) is heated to about  $90^{\circ}\text{C}$  -  $100^{\circ}\text{C}$  for about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to  $54^{\circ}\text{C}$ , which is preferable for

15 primer hybridization. The synthesis reaction may occur at room temperature up to a temperature above which the polymerase (inducing agent) no longer functions efficiently. Thus, for example, if DNA polymerase is used as inducing agent, the temperature

20 is generally no greater than about  $40^{\circ}\text{C}$ . An exemplary PCR buffer comprises the following: 50 mM KCl; 10 mM Tris-HCl at pH 8.3; 1.5 mM  $\text{MgCl}_2$ ; 0.001% (wt/vol) gelatin, 200  $\mu\text{M}$  dATP; 200  $\mu\text{M}$  dTTP; 200  $\mu\text{M}$  dCTP; 200  $\mu\text{M}$  dGTP; and 2.5 units *Thermus aquaticus* DNA polymerase I

25 (U.S. Patent No. 4,889,818) per 100 microliters of buffer. PCR for amplifying the recombinant penton base and fiber proteins of this invention was performed as described in Example 1.

30 The inducing agent may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other

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available DNA polymerases, reverse transcriptase, and other enzymes, including heat-stable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be inducing agents, however, which initiate synthesis at the 5' end and proceed in the above direction, using the same process as described above.

The inducing agent also may be a compound or system which will function to accomplish the synthesis of RNA primer extension products, including enzymes. In preferred embodiments, the inducing agent may be a DNA-dependent RNA polymerase such as T7 RNA polymerase, T3 RNA polymerase or SP6 RNA polymerase. These polymerases produce a complementary RNA polynucleotide. The high turn-over rate of the RNA polymerase amplifies the starting polynucleotide as has been described by Chamberlin et al., The Enzymes, ed. P. Boyer, PP. 87-108, Academic Press, New York (1982). Another advantage of T7 RNA polymerase is that mutations can be introduced into the polynucleotide synthesis by replacing a portion of cDNA with one or more mutagenic oligodeoxynucleotides (polynucleotides) and transcribing the partially-mismatched template directly as has been previously described by Joyce et al., Nuc. Acid Res. 17: 711-722 (1989). Amplification systems based on transcription have been described by Gingeras et al., in PCR Protocols, A Guide to Methods and Applications, pp 245-252, Academic Press, Inc., San Diego, CA (1990).

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If the inducing agent is a DNA-dependent RNA polymerase and therefore incorporates ribonucleotide triphosphates, sufficient amounts of ATP, CTP, GTP and UTP are admixed to the primer extension reaction admixture and the resulting solution is treated as described above.

The newly synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which can be used in the succeeding steps of the process.

After producing various polypeptide-encoding DNA homologs for one or a plurality of different genes or DNA segments, the DNA molecules are typically further amplified. While the DNA molecules can be amplified by classic techniques such as incorporation into an autonomously replicating vector, it is preferred to first amplify the molecules by subjecting them to a polymerase chain reaction (PCR) prior to inserting them into a vector. PCR is typically carried out by thermocycling i.e., repeatedly increasing and decreasing the temperature of a PCR reaction admixture within a temperature range whose lower limit is about 10°C to about 40°C and whose upper limit is about 90°C to about 100°C. The preferred amplification procedure was performed as described in Example 1. The increasing and decreasing can be continuous, but is preferably phasic with time periods of relative temperature stability at each of temperatures favoring polynucleotide synthesis, denaturation and hybridization.

PCR amplification methods are described in detail in U.S. Patent Nos. 4,683,192, 4,683,202, 4,800,159, and 4,965,188, and at least in several texts including "PCR Technology: Principles and Applications for DNA Amplification", H. Erlich, ed., Stockton Press, New

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York (1989); and "PCR Protocols: A Guide to Methods and Applications", Innis et al., eds., Academic Press, San Diego, California (1990). Various preferred methods and primers used herein are described hereinafter and are also described in Nilsson, et al., Cell 58: 707 (1989), Ennis, et al., PNAS USA 87: 2833-7 (1990), and Zemmour, et al., Immunogenetics 33: 310-20 (1991), for example. In particular for amplifying the therapeutic nucleotide sequences for use in this invention, it is preferred to design primers from comparison of 5' and 3' untranslated regions of known allelic forms, with selection of conserved sequences. Restriction sites may also be incorporated into the 5' and 3' primers to enable the amplification products to be subcloned into sequencing or expression vectors. It may also be helpful to place a 4-base spacer sequence proximal to the restriction site to improve the efficiency of cutting amplification products with enzymes.

The following primers are preferred for amplification of penton base and fiber cDNA from adenovirus type 2 DNA, preferably in separate reactions. The adenoviral template DNA is obtained as described in Example 1. Resulting cDNAs may then be cloned and sequenced as described herein. These primers are appropriate for use in amplifying all known and presently unknown types of adenovirus penton base and fiber.

The 5' and 3' primers used to amplify the penton base were, respectively, 5'-TTTCTAGAAGTATGCAGCGCGCG-3' (SEQ ID NO 2) and 5'-TTTCTAGATCAAAAAGTGCGGCT-3' (SEQ ID NO 3). The 5' and 3' primers used to amplify the fiber were, respectively, 5'-AAAGGATCCAGCTGATGAAACGCGCCA-3' (SEQ ID NO 4) and 5'-TTTGGTACCAGCTGTTATTCCTGGGCA-3' (SEQ ID NO 5). The

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restriction endonuclease cloning sites indicated by the underlined nucleotides are described in Example 1.

In preferred embodiments only one pair of first and second primers is used per amplification reaction. The amplification reaction products obtained from a plurality of different amplifications, each using a plurality of different primer pairs, are then combined. However, the present invention also contemplates DNA homolog production via co-amplification (using two pairs of primers), and multiplex amplification (using up to about 8, 9 or 10 primer pairs).

## 2. Expression Vector Systems for Expression of Penton Base and Fiber Proteins

Expression of recombinant penton base and fiber proteins of this invention is accomplished through the use of expression vectors into which the PCR amplified penton base or fiber sequences described above have been inserted. The expression vectors may be constructed utilizing any of the well-known vector construction techniques. Those techniques, however, are modified to the extent that the translatable nucleotide sequence to be inserted into the genome of the host cell is flanked "upstream" of the sequence by an appropriate promoter and/or enhancer sequences.

In a preferred embodiment, the vector also contains a selectable marker. After expression, the product of the translatable nucleotide sequence may then be purified using antibodies against that sequence. One example of a selectable marker is neomycin resistance. A plasmid encoding neomycin resistance, such as *phshsneo*, *phsneo*, or *pcopneo*, may be included in each transfection such that a population of cells that express the gene(s) of choice may be ascertained by growing the transfectants in

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selection medium.

In a preferred embodiment, the translatable nucleotide sequence may be incorporated into a plasmid with an appropriate controllable transcriptional promoter, translational control sequences, and a polylinker to simplify insertion of the translatable nucleotide sequence in the correct orientation, and may be expressed in the host cells. Various host cells include a eucaryotic insect cell, such as *Spodoptera frugiperda*, or a procaryotic cell, such as *E. coli*. Preferably, there are 5' control sequences defining a promoter for initiating transcription and a ribosome binding site operatively linked at the 5' terminus of the upstream translatable DNA sequence.

To achieve high levels of gene expression in transformed or transfected cells -- for example, *E. coli* -- it is necessary to use not only strong promoters to generate large quantities of mRNA, but also ribosome binding sites to ensure that the mRNA is efficiently translated. In *E. coli*, for example, the ribosome binding site includes an initiation codon (AUG) and a sequence 3-9 nucleotides long located 3-11 nucleotides upstream from the initiation codon (Shine et al., Nature, 254:34 (1975)). The sequence, AGGAGGU, which is called the Shine-Dalgarno (SD) sequence, is complementary to the 3' end of *E. coli* 16S mRNA. Binding of the ribosome to mRNA and the sequence at the 3' end of the mRNA can be affected by several factors, including (1) the degree of complementarity between the SD sequence and 3' end of the 16S tRNA; and (2) the spacing and possibly the DNA sequence lying between the SD sequence and the AUG. (See, e.g., Roberts et al., PNAS USA 76: 760 (1979a); Roberts et al., PNAS USA 76: 5596 (1979b); Guarente et al., Science 209: 1428 (1980); and Guarente et al.,

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Cell 20: 543 (1980).)

Optimization is generally achieved by measuring the level of expression of genes in plasmids in which this spacing is systematically altered. Comparison of  
5 different mRNAs shows that there are statistically preferred sequences from positions -20 to +13 (where the A of the AUG is position 0; see, e.g., Gold et al., Ann. Rev. Microbiol. 35: 365 (1981). Leader sequences have also been shown to influence  
10 translation dramatically (Roberts et al., 1979 a, b *supra*). Binding of the ribosome may also be affected by the nucleotide sequence following the AUG, which affects ribosome binding. (See, e.g., Taniguchi et al., J. Mol. Biol. 118: 533 (1978).)

15 Vectors for use in producing large quantities of the recombinant adenovirus-derived proteins of this invention, penton base and fiber, are designed for the expression of proteins in bacteria, in mammalian cells or in insect cells. For expression in bacterial *E.*  
20 *coli*, the expression vectors are preferably utilized in conjunction with bacterial "host" cells adapted for the production of useful quantities of proteins or polypeptides. Such vectors may include a procaryotic replicon i.e., a nucleotide sequence having the  
25 ability to direct autonomous replication and maintenance of the recombinant DNA molecule extra-chromosomally in a procaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition,  
30 those embodiments that include a procaryotic replicon may also include a gene whose expression confers a selective advantage, such as drug resistance, to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer  
35 resistance to ampicillin or tetracycline. Vectors

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typically also contain convenient restriction sites for insertion of translatable nucleotide sequences.

5       The procaryotic expression vectors also contain promoters which can be used in the microbial organism for expression of its own proteins. Those promoters most commonly used include the beta-lactamase and lactose promoter systems and the tryptophan promoter system as described in the European Patent Application No. 0125023.

10       Exemplary procaryotic expression vectors include the plasmids pUC8, pUC9, pUC18, pBR322, and pBR329 available from BioRad Laboratories (Richmond, CA), pPL and pKK223 available from Pharmacia (Piscataway, NJ), and pBS and M13mp19 (Stratagene, La Jolla, CA). Other  
15       exemplary vectors include pCMU (Nilsson, et al., Cell 58: 707 (1989)). Other appropriate vectors may also be synthesized, according to known methods; for example, vectors pCMU/K<sup>b</sup> and pCMUII used in various applications herein are modifications of pCMUIV  
20       (Nilsson, et al., *supra*).

      Mammalian expression vector systems are also contemplated for the expression of recombinant penton base and fiber proteins for use in this invention. For controlling expression in mammalian cells,  
25       viral-derived promoters are most commonly used. For example, frequently used promoters include polyoma, adenovirus type 2, and Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily  
30       from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 base pair sequence extending from the Hind III restriction site toward



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the Bgl I site located in the viral origin of replication. Also contemplated is using the promoter sequences normally associated with the desired sequence for expression, in this instance, adenovirus

5 2. Origins of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral sources such as polyoma and adenovirus or may be provided by the host cell chromosomal replication

10 mechanism. The latter is sufficient for integration of the expression vector in the host cell chromosome.

The preferred expression vector system for use preparing large quantities of recombinant penton base and fiber proteins is the baculovirus expression

15 vector system, the details of which are described in Example 1. It will be understood that this invention, although described herein in terms of a preferred embodiment, should not be construed as limited to the host cells, expression vectors and expression vectors

20 systems exemplified. Other expression vector systems, well known to one of ordinary skill in the art and described by Kaufman et al., In "Current Protocols in Molecular Biology", Ausubel et al., eds., Unit 16, New York (1990), are contemplated for preparing

25 recombinant penton base and fiber proteins for use in this invention.

### 3. Expression and Purification of Recombinant Penton Base and Fiber Proteins

To express the recombinant penton base and

30 fiber proteins for use in this invention, the expression vectors containing the nucleotide sequences that encode the preferred proteins are transfected into the recipient hosts cells described above. The host cell can be either procaryotic or eucaryotic.

35 Bacterial cells are preferred procaryotic host cells

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and typically are a strain of *E. coli* such as the MC1061 or JM109 strains. Preferred eucaryotic host cells include yeast, insect and mammalian cells, where the latter are those from mouse, rat, monkey or human fibroblastic cell lines. Preferred eucaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61 and NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL1658. Most preferred for the expression of the recombinant penton base and fiber proteins is an insect cell, which include *Spodoptera frugiperda* and *Trichoplusia ni* strains as described in Example 1.

Transfection may be accomplished via numerous methods, depending on the type of vector used, including the calcium phosphate method, the DEAE-dextran method, the stable transfer method, electroporation, or via the liposome mediation method. Numerous texts are available which set forth known transfection methods and other procedures for introducing nucleotides into cells; see, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, NY (1991). The preferred methods of transfection are performed as described in Example 1.

Successfully transformed cells, i.e., cells that contain the penton base or fiber expression vectors, are usually monitored by an appropriate immunological, functional or visual assay. For example, with the baculovirus vector expression vector system transfected into host insect cells, the screening for successfully transfected cells is accomplished by visual inspection in a plaque assay for the presence or absence of occlusion bodies as described in Example 1. Wild-type viral plaques contain the occlusions whereas recombinant transfected viral plaques lack occlusions.

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In addition to the direct assaying for the presence of recombinants, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of a protein of this invention. For example, samples of a culture containing cells suspected of being transformed are harvested and assayed for the subject expressed protein of this invention using either monoclonal or polyclonal antibodies that are specific for the expressed protein, such as anti-penton base or anti-fiber antibodies.

Purification of the expressed recombinant penton base and fiber proteins is accomplished by a variety of techniques depending on the selected expression vector system. For the preferred baculovirus expression vector system for use in obtaining large quantities of recombinant penton base and fiber proteins, following the purification of recombinant virus and preparation of high titer viral stocks, the viral stock containing the recombinant vector is used to infect host insect cells as described in Example 1. The constitutively expressed recombinant proteins are then purified by lysing cells with hypotonic buffer and collecting the intracellular proteins by centrifugation.

Purified recombinant penton base has a number of properties which make it ideal for gene therapy applications. For example, penton base is capable of saturable and specific binding to human epithelial cells. The affinity value is about  $K_d = 55 \text{ nM}$ , or approximately  $8.9 \times 10^4$  sites per cell. Binding occurs at sites of membrane contact with the cell substratum. Binding to epithelial cells is

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magnesium-dependent. Penton base binding to epithelial cells is 5-10 fold greater than binding to fibroblasts. Binding of penton base to epithelial cells is increased 10-fold at low pH.

5 D. Systems for the Expression of Therapeutic Nucleotide Sequences Following Gene Transfer Mediated by Adenovirus-derived Proteins

1. Nucleic Acid Segments

10 A therapeutic nucleotide composition of the present invention comprises a nucleotide sequence encoding a therapeutic molecule as described in Section F below. A therapeutic nucleotide composition may further comprise an enhancer element or a promoter located 5' to and controlling the expression of said  
15 therapeutic nucleotide sequence or gene. The promoter is a DNA segment that contains a DNA sequence that controls the expression of a gene located 3' or downstream of the promoter. The promoter is the DNA sequence to which RNA polymerase specifically binds  
20 and initiates RNA synthesis (transcription) of that gene, typically located 3' of the promoter.

The subject therapeutic nucleotide composition consists of a nucleic acid molecule that comprises at least 2 different operatively linked DNA segments.  
25 The DNA can be manipulated and amplified by PCR as described in Section C and by using the standard techniques described in Molecular Cloning: A Laboratory Manual, 2nd Edition, Maniatis et al., eds., Cold Spring Harbor, New York (1989). Typically, to  
30 produce a therapeutic nucleotide composition of the present invention, the sequence encoding the selected therapeutic composition and the promoter or enhancer are operatively linked to a vector DNA molecule capable of autonomous replication in a cell either in  
35 vivo or in vitro. By operatively linking the enhancer

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element or promoter and the nucleotide sequence encoding the therapeutic nucleotide composition to the vector, the attached segments are replicated along with the vector sequences. Thus, a recombinant DNA molecule (rDNA) of the present invention is a hybrid DNA molecule comprising at least 2 nucleotide sequences not normally found together in nature.

The therapeutic nucleotide composition of the present invention is from about 20 base pairs to about 100,000 base pairs in length. Preferably the nucleic acid molecule is from about 50 base pairs to about 50,000 base pairs in length. More preferably the nucleic acid molecule is from about 50 base pairs to about 10,000 base pairs in length. Most preferred is a nucleic acid molecule from about 50 pairs to about 4,000 base pairs in length. The therapeutic nucleotide can be a gene or gene fragment that encodes a protein or peptide that provides the desired therapeutic effect such as replacement of alpha 1-antitrypsin or cystic fibrosis transmembrane regulator protein and the like. Alternatively, the therapeutic nucleotide can be a DNA or RNA oligonucleotide sequence that exhibits enzymatic therapeutic activity. Examples of the latter include antisense oligonucleotides that will inhibit the transcription of deleterious genes or ribozymes that act as site-specific ribonucleases for cleaving selected mutated gene sequences. In another variation, a therapeutic nucleotide sequence of the present invention may comprise a DNA construct capable of generating therapeutic nucleotide molecules, including ribozymes and antisense DNA, in high copy numbers in target cells, as described in published PCT application No. WO 92/06693 (the disclosure of which is incorporated herein by reference).

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A regulatable promoter is a promoter where the rate of RNA polymerase binding and initiation is modulated by external stimuli. Such stimuli include compositions light, heat, stress and the like.

5 Inducible, suppressible and repressible promoters are regulatable promoters. Regulatable promoters may also include tissue specific promoters. Tissue specific promoters direct the expression of that gene to a specific cell type. Tissue specific promoters cause  
10 the gene located 3' of it to be expressed predominantly, if not exclusively in the specific cells where the promoter expressed its endogenous gene. Typically, it appears that if a tissue-specific promoter expresses the gene located 3' of it at all,  
15 then it is expressed appropriately in the correct cell types as has been reviewed by Palmiter et al., Ann. Rev. Genet. 20: 465-499 (1986).

When a tissue specific promoter controls the expression of a gene, that gene will be expressed in a  
20 small number of tissues or cell types rather than in substantially all tissues and cell types. Examples of tissue specific promoters include the immunoglobulin promoter described by Brinster et al., Nature 306: 332-336 (1983) and Storb et al., Nature 310: 238-231  
25 (1984); the elastase-I promoter described by Swift et al., Cell 38: 639-646 (1984); the globin promoter described by Townes et al., Mol. Cell. Biol. 5: 1977-1983 (1985), and Magram et al., Mol. Cell. Biol. 9: 4581-4584 (1989), the insulin promoter described by  
30 Bucchini et al., PNAS USA, 83: 2511-2515 (1986) and Edwards et al., Cell 58: 161 (1989); the immunoglobulin promoter described by Ruscon et al., Nature 314: 330-334 (1985) and Grosscheld et al., Cell 38: 647-658 (1984); the alpha actin promoter described  
35 by Shani, Mol. Cell. Biol. 6: 2624-2631 (1986); the

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alpha crystalline promoter described by Overbeek et al., PNAS USA 82: 7815-7819 (1985); the prolactin promoter described by Crenshaw et al., Genes and Development 3: 959-972 (1989); the proopiomelanocortin  
5 promoter described by Tremblay et al., PNAS USA 85: 8890-8894 (1988); the beta-thyroid stimulating hormone (BTSN) promoter described by Tatsumi et al., Nippon Rinsho 47: 2213-2220 (1989); the mouse mammary tumor virus (MMTV) promoter described by Muller et al., Cell  
10 54: 105 (1988); the albumin promoter described by Palmiter et al., Ann. Rev. Genet. 20: 465-499 (1986); the keratin promoter described by Vassar et al., PNAS USA 86: 8565-8569 (1989); the osteonectin promoter described by McVey et al., J. Biol. Chem. 263:  
15 11,111-11,116 (1988); the prostate-specific promoter described by Allison et al., Mol. Cell. Biol. 9: 2254-2257 (1989); the opsin promoter described by Nathans et al., PNAS USA 81: 4851-4855 (1984); the olfactory marker protein promoter described by  
20 Danciger et al., PNAS USA 86: 8565-8569 (1989); the neuron-specific enolase (NSE) promoter described by Forss-Pelter et al., J. Neurosci. Res. 16: 141-151 (1986); the L-7 promoter described by Sutcliffe, Trends in Genetics 3: 73-76 (1987) and the protamine 1  
25 promoter described Peschon et al., Ann. New York Acad. Sci. 564: 186-197 (1989) and Braun et al., Genes and Development 3: 793-802 (1989).

## 2. Expression Vector Systems

The introduction of exogenous DNA into  
30 eucaryotic cells has become one of the most powerful tools of the molecular biologist. The term "exogenous" encompasses any therapeutic composition of this invention which is administered by the therapeutic methods of this invention. Thus,  
35 "exogenous" is also referred to as "foreign,

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nonnative, and the like". The methods of this invention requires efficient delivery of the DNA into the nucleus of the recipient cell and subsequent identification of cells that are expressing the foreign DNA.

Engineered vectors such as plasmids or bacteriophages (phages) or other DNA sequences that are able to replicate in a host cell can be used to construct cells that act as factories to produce large amounts of specific viral proteins. Recombinant plasmids will be used herein as exemplary vectors, also called cloning vehicles. See U.S. Patent No. 4,338,397, incorporated herein by reference.

Plasmids are extrachromosomal genetic elements found in a variety of bacterial species. They are typically double-stranded, closed, circular DNA molecules. A widely-used plasmid is pBR322, a vector whose nucleotide sequence and endonuclease cleavage sites are well known.

Nucleic acid production using plasmid or phage vectors has become very straightforward. The plasmid or phage DNA is cleaved with a restriction endonuclease and joined *in vivo* to a foreign DNA of choice. The resulting recombinant plasmid or phage is then introduced into a cell such as *E. coli*, and the cell so produced is induced to produce many copies of the engineered vector. Once a sufficient quantity of DNA is produced by the cloning vector, the produced foreign DNA is excised and placed into a second vector to produce or transcribe the protein or polypeptide encoded by the foreign gene.

Depending on the DNA (intact gene, cDNA, or bacterial gene), it may be necessary to provide eucaryotic transcription and translation signals to direct expression in recipient cells either *in vivo* or



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in vitro. These signals may be provided by combining the foreign DNA *in vitro* with an expression vector.

Expression vectors contain sequences of DNA that are required for the transcription of cloned genes and the translation of their messenger RNA's (mRNA's) into proteins. Typically, such required sequences or control elements are: (1) a promoter that signals the starting point for transcription; (2) a terminator that signals the ending point of transcription; (3) an operator that regulates the promoter; (4) a ribosome binding site for the initial binding of the cells' protein synthesis machinery; and (5) start and stop codons that signal the beginning and ending of protein synthesis.

To be useful, an expression vector should possess several additional properties. It should be relatively small and contain a strong promoter. The expression vector should carry one or more selectable markers to allow identification of transformants. It should also contain a recognition site for one or more restriction enzymes in regions of the vector that are not essential for expression.

The construction of expression vectors is, therefore, a complicated and somewhat unpredictable venture. The only true test of the effectiveness of an expression vector is to measure the frequency with which the synthesis of the appropriate mRNA is initiated. However, quantitation of mRNA is tedious, and it is often difficult to obtain accurate measurements. Other more practicable means have, therefore, been developed to detect transformation.

One such means has been to monitor synthesis of foreign proteins in transformed cells with enzymatic assays. Several marker genes have been developed for indicating that transformation has occurred.

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Another means used to monitor transformation involves the use of immunological reagents. If the level of expressed protein is sufficiently high, then cytoplasmic or surface immunofluorescence with an antibody conjugated to a fluorescent dye such as fluorescein or rhodamine may be used to detect vector-specific protein expression products.

More commonly, transformed cells are cultured in the presence of radioactivity after immunoprecipitation. This approach has used *Staphylococcus aureus* protein A selection of immune complexes (Kessler, J. Immunol. 115: 1617-1624 (1975)) and the Western blotting procedure (Renart et al., PNAS USA 76: 3116-3120 (1979)) to detect transformation-specific markers.

A vector of the present invention is a nucleic acid (preferably DNA) molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. In the present invention, one of the nucleotide segments to be operatively linked to vector sequences encodes at least a portion of a therapeutic molecule. Preferably, the entire peptide-coding sequence of the therapeutic gene is inserted into the vector and expressed; however, it is also feasible to construct a vector which also includes some non-coding sequences as well. Preferably, the non-coding sequences are excluded. Alternatively, a nucleotide sequence for a soluble form of a polypeptide may be utilized. Another preferred vector includes a nucleotide sequence encoding at least a portion of a therapeutic nucleotide sequence operatively linked to the vector for expression.

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As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting between different genetic environments another nucleic acid to which it has been operatively linked. Preferred  
5 vectors are those capable of autonomous replication and expression of structural gene products present in the nucleotide (DNA) segments to which they are operatively linked.

As used herein with regard to DNA sequences or  
10 segments, the phrase "operatively linked" means the sequences or segments have been covalently joined into one piece of DNA, whether in single or double stranded form.

The choice of vector into which a therapeutic  
15 nucleotide sequence of this invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., vector replication and protein expression, and the host cell to be transformed, these being limitations inherent in  
20 the art of constructing recombinant DNA molecules.

In various embodiments, a vector is utilized for the production of therapeutic proteins or polypeptides useful in the present invention. Such vectors are preferably utilized in conjunction with eucaryotic  
25 cells. Such vectors may include a eucaryotic replicon i.e., a nucleotide sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extra-chromosomally or stably in a eucaryotic recipient cell. Such replicons are  
30 well known in the art. In addition, those embodiments that include a eucaryotic replicon may also include a gene whose expression confers a selective advantage, such as drug resistance. Vectors typically also contain convenient restriction sites for insertion of  
35 translatable nucleotide sequences. Exemplary vectors

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include the Chinese hamster ovary cell expression vectors, vaccinia virus expression system, vaccinia virus/T7 RNA polymerase hybrid system and the like as described by Kaufman et al., In "Current Protocols in Molecular Biology", Ausubel et al., eds., Unit 16.12.5, New York (1990).

A sequence of nucleotides adapted for directional ligation, i.e., a polylinker, is a region of the expression vector that (1) operatively links for replication and transport the upstream and downstream nucleotide sequences and (2) provides a site or means for directional ligation of a nucleotide sequence into the vector. Typically, a directional polylinker is a sequence of nucleotides that defines two or more restriction endonuclease recognition sequences, or restriction sites. Upon restriction cleavage, the two sites yield cohesive termini to which a translatable nucleotide sequence can be ligated to the expression vector. Preferably, the two restriction sites provide, upon restriction cleavage, cohesive termini that are non-complementary and thereby permit directional insertion of a translatable nucleotide sequence into the vector. In one embodiment, the directional ligation means is provided by nucleotides present in the upstream nucleotide sequence, downstream nucleotide sequence, or both. In another embodiment, the sequence of nucleotides adapted for directional ligation comprises a sequence of nucleotides that defines multiple directional cloning means. Where the sequence of nucleotides adapted for directional ligation defines numerous restriction sites, it is referred to as a multiple cloning site.

A translatable nucleotide sequence is a linear series of nucleotides that provide an uninterrupted series of at least 8 codons that encode a polypeptide

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in one reading frame. Preferably, the nucleotide sequence is a DNA sequence. The vector itself may be of any suitable type, such as a viral vector (RNA or DNA), naked straight-chain or circular DNA, or a vesicle or envelope containing the nucleic acid material and any polypeptides that are to be inserted into the cell. With respect to vesicles, techniques for construction of lipid vesicles, such as liposomes described in Section E, are well known. Such liposomes may be targeted to particular cells using other conventional techniques, such as providing an antibody or other specific binding molecule on the exterior of the liposome. See, e.g., A. Huang, et al., J. Biol. Chem. 255: 8015-8018 (1980).

Most useful vectors contain multiple elements including one or more of the following, depending on the nature of the recipient cell: an SV40 origin of replication for amplification to high copy number; an efficient promoter element for high-level transcription initiation; mRNA processing signals such as mRNA cleavage and polyadenylation sequences (and frequently, intervening sequences as well); polylinkers containing multiple restriction endonuclease sites for insertion of foreign DNA; selectable markers that can be used to select cells that have stably integrated the plasmid DNA; and plasmid replication control sequences to permit propagation in bacterial cells. In addition to the above, many vectors also contain an inducible expression system that is regulated by an external stimulus. Sequences from a number of promoters that are required for induced transcription have been identified and engineered into expression vectors to obtain inducible expression. Several useful inducible vectors have been based on induction by  $\gamma$ -interferon,

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heat-shock, heavy metal ions, and steroids (e.g. glucocorticoids). (See, e.g., Kaufman, Meth. Enzymol. 185: 487-511 (1990).) Other promoters contemplated for use in this invention are described in Example 5.

5           A preferred vector in which therapeutic nucleotide compositions of this invention are present is a plasmid; more preferably, it is a high-copy-number plasmid. It is also desirable that the vector contain an inducible promoter sequence, as  
10           inducible promoters tend to limit selection pressure against cells into which such vectors (which are often constructed to carry non-native or chimeric nucleotide sequences) have been introduced by the adenovirus-derived proteins of this invention. It is  
15           also preferable that the vector of choice be best suited for expression in the preselected recipient cell type depending on the nature of the gene replacement therapy.

          A tissue containing a therapeutic nucleotide  
20           sequence of the present invention may also be produced by directly introducing the vector containing the sequence into an animal or by linking the therapeutic oligonucleotide directly to the adenovirus-derived proteins. Direct vector delivery in vivo may be  
25           accomplished by transducing the desired cells and tissues with viral vectors or other physical gene transfer vehicles. Other physical agents including naked plasmids, cloned genes encapsulated in targetable liposomes (see Section E below) or in  
30           erythrocyte ghosts have been use to introduce genes, proteins, toxins and other agents directly into whole animals.

          Direct injection of therapeutic nucleotide sequences, with or without penton -- but preferably in  
35           conjunction therewith -- is also a viable alternative

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for delivery of therapeutic sequences. For example, the injection of naked calcium phosphate-precipitated plasmid into rat liver and rat spleen or a protein-coated plasmid into the portal vein has resulted in gene expression of the foreign gene the rat livers. (See, e.g., Kaneda, et al., Science, 243:375 (1989), incorporated by reference herein.)

E. Liposomes

In preferred embodiments, a subject therapeutic sequence, penton base, fiber or penton complex, penton conjugates and compositions of the present invention (hereinafter, "active ingredient") is covalently linked to a carrier, such as a phospholipid. More preferably, the active ingredient is removably inserted into a liposome, i.e., incorporated (anchored) into the liposome bilayer via the LDL binding moiety. (See, e.g., Gregoriadis, Trends in Biotech., 3:235-241 (1985) and Eriksson et al., in Liposome Technology Vol. II, G. Gregoriadis (ed.), CRC Press, Boca Raton, FL, pp. 141-156. The disclosures of these articles are incorporated herein by reference.) Since the penton base does not typically include a transmembrane anchor sequence, it is necessary to construct a lipid binding site on the virus protein in order that it may associate with liposomes.

Therapeutic nucleotide sequences and compositions according to the present invention can be administered in a liposome (micelle) formulation which can be administered by application to mucous membranes of body cavities. Juliano et al., J. Pharmacol. Exp. Ther. 214: 381 (1980). Liposomes are prepared by a variety of techniques well known to those skilled in the art to yield several different physical structures, ranging from the smallest unilamellar

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vesicles of approximately 20 to 50 nanometers in diameter up to multilamellar vesicles of tens of microns in diameter. Gregoriadis (ed.), Liposome Technology 1, CRC Press (1984). Therapeutic

5 nucleotide sequences and compositions for nasal formulations are preferably hydrated with a lyophilized powder of multilamellar vesicles to form liposomes containing the sequences and compositions according to the present invention.

10 Therapeutic sequences, penton base, fiber or penton complex, penton conjugates and compositions of the present invention (hereinafter, "active ingredient") may also be incorporated into liposomal vesicles via reverse loading (see U.S. Pat. No. 15 5,104,661), or in the manner described for the incorporation of amphotericin B into lipid vesicles. (See, e.g., Lopez-Berenstein, et al., J. Infect. Dis. 151: 704-710 (1985); Lopez-Berenstein, Antimicrob. Agents Chemother. 31: 675-8 (1987); Lopez-Berenstein, 20 et al., J. Infect. Dis. 150: 278-283 (1984); and Mehta, et al., Biochem. Biophys. Acta 770: 230-4 (1984).) Delivery may also be accomplished using liposomes with enhanced circulation time (see U.S. Pat. No. 5,013,556). Use of liposomes to deliver 25 therapeutic nucleotide sequences according to the present invention may also be utilized to regulate expression of target sequences or to limit the proliferation of virus or retrovirus. (See, e.g., published PCT application No. WO 92/06192, 30 incorporated herein by reference.)

The amount of active ingredient incorporated into liposomes may be about 0.1  $\mu$ g active ingredient per mg lipid to about 1 mg per mg lipid. The dosage amount of active ingredient administered in lipid 35 encapsulated form is preferably about 0.1-1mg per mg



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lipid.

Direct vector delivery in vivo may be accomplished via liposomal delivery. Various physical agents including therapeutic nucleotide sequences encapsulated in targetable liposomes have been use to introduce genes, proteins, toxins and other agents directly into whole animals. See, for example, the liposome-mediated gene delivery in vivo and expression of preproinsulin genes in recipient rats described by Nicolaou, et al., PNAS USA 80:1068 (1983) and Soriano, et al., PNAS USA 80:7128 (1983). All cited disclosures are incorporated herein by reference.

F. Therapeutic Sequences and Compositions

In various alternative embodiments of the present invention, therapeutic sequences and compositions useful for practicing the therapeutic methods described herein are contemplated. Therapeutic compositions of the present invention may contain a physiologically tolerable carrier together with one or more therapeutic nucleotide sequences of this invention, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the composition is not immunogenic or otherwise able to cause undesirable side effects when administered to a mammal or human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

For example, the present invention comprises

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therapeutic compositions useful in the specific targeting of epithelial or non-epithelial cells as well as in delivering a therapeutic nucleotide sequence to those cells. Therapeutic compositions  
5 designed to preferentially target to epithelial cells may comprise an adenovirus-derived protein and a therapeutic nucleotide sequence. As described hereinabove, penton base or penton complex are preferred adenovirus-derived proteins useful in the  
10 presently-disclosed therapeutic compositions and methods.

Compositions designed to preferentially target non-epithelial cells may include an adenovirus-derived protein-ligand conjugate and a therapeutic nucleotide  
15 sequence. Examples of useful ligands directed to specific receptors (identified in parentheses) include the V3 loop of HIV gp120 (CD4); transferrin (transferrin receptor); LDL (LDL receptors); and deglycosylated proteins (asialoglycoprotein receptor).  
20 Polypeptides having a sequence that includes an amino acid residue sequence selected from the group comprising -EDPGFFNVE- (SEQ ID NO 6) and -EDPGKQLYNVE- (SEQ ID NO 7) are capable of targeting receptors such as the CR2 receptor, and are thus useful in  
25 compositions disclosed herein.

Useful ligands also include antibodies and attachment sequences, as well as receptors themselves. Antibodies to cell receptor molecules such as integrins and the like, MHC Class I and Class II,  
30 asialoglycoprotein receptor, transferrin receptors, LDL receptors, CD4, and CR2 are but a few useful according to the present invention. It is also understood that the ligands typically bound by receptors, as well as analogs to those ligands, may be  
35 used as cellular targeting agents as disclosed herein.

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Exemplary and preferred nucleotide sequences encode an expressible peptide, polypeptide or protein, and may further include an active constitutive or inducible promoter sequence. For example, preferred  
5 therapeutic nucleotide sequences according to the present invention are capable of delivering HIV antisense nucleotides to latently-infected T cells via CD4. Similarly, delivery of Epstein-Barr Virus (EBV) EBNA-1 antisense nucleotides to B cells via CR2 is  
10 capable of effecting therapeutic results.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as  
15 injectables either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified, or formulated into suppositories, ointments, creams,  
20 dermal patches, or the like, depending on the desired route of administration.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts  
25 suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof, including vegetable oils, propylene glycol, polyethylene glycol and benzyl  
30 alcohol (for injection or liquid preparations); and vaseline, vegetable oil, animal fat and polyethylene glycol (for externally applicable preparations). In addition, if desired, the composition can contain wetting or emulsifying agents, isotonic agents,  
35 dissolution promoting agents, stabilizers, colorants,

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antiseptic agents, soothing agents and the like additives (as usual auxiliary additives to pharmaceutical preparations), pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic compositions of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

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A therapeutic composition typically contains an amount of a therapeutic nucleotide sequence of the present invention sufficient to deliver a therapeutically effective amount to the target tissue, typically an amount of at least 0.1 weight percent to about 90 weight percent of therapeutic nucleotide sequence per weight of total therapeutic composition. A weight percent is a ratio by weight of therapeutic nucleotide sequence to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of DNA segment per 100 grams of total composition.

The therapeutic nucleotide compositions comprising synthetic oligonucleotide sequences of the present invention can be prepared using any suitable method, such as, the phosphotriester or phosphodiester methods. See Narang et al., Meth. Enzymol., 68:90, (1979); U.S. Patent No. 4,356,270; and Brown et al., Meth. Enzymol., 68:109, (1979).

For therapeutic oligonucleotides sequence compositions in which a family of variants is preferred, the synthesis of the family members can be conducted simultaneously in a single reaction vessel, or can be synthesized independently and later admixed in preselected molar ratios.

For simultaneous synthesis, the nucleotide residues that are conserved at preselected positions of the sequence of the family member can be introduced in a chemical synthesis protocol simultaneously to the variants by the addition of a single preselected nucleotide precursor to the solid phase oligonucleotide reaction admixture when that position number of the oligonucleotide is being chemically added to the growing oligonucleotide polymer. The addition of nucleotide residues to those positions in the sequence that vary can be introduced

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simultaneously by the addition of amounts, preferably equimolar amounts, of multiple preselected nucleotide precursors to the solid phase oligonucleotide reaction admixture during chemical synthesis. For example, where all four possible natural nucleotides (A,T,G and C) are to be added at a preselected position, their precursors are added to the oligonucleotide synthesis reaction at that step to simultaneously form four variants.

This manner of simultaneous synthesis of a family of related oligonucleotides has been previously described for the preparation of "degenerate oligonucleotides" by Ausubel et al, in "Current Protocols in Molecular Biology", Suppl. 8. p.2.11.7, John Wiley & Sons, Inc., New York (1991), and can readily be applied to the preparation of the therapeutic oligonucleotide compositions described herein.

Nucleotide bases other than the common four nucleotides (A,T,G or C), or the RNA equivalent nucleotide uracil (U), can be used in the present invention. For example, it is well known that inosine (I) is capable of hybridizing with A, T and G, but not C.

Thus, where all four common nucleotides are to occupy a single position of a family of oligonucleotides, that is, where the preselected therapeutic nucleotide composition is designed to contain oligonucleotides that can hybridize to four sequences that vary at one position, several different oligonucleotide structures are contemplated. The composition can contain four members, where a preselected position contains A,T,G or C. Alternatively, the composition can contain two members, where a preselected position contains I or C,

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and has the capacity the hybridize at that position to all four possible common nucleotides. Finally, other nucleotides may be included at the preselected position that have the capacity to hybridize in a non-destabilizing manner with more than one of the common nucleotides in a manner similar to inosine.

G. Therapeutic Methods

Various therapeutic methods are also contemplated by the present invention. For example, it has now been discovered that proteins derived from adenovirus are capable of delivering a therapeutic nucleotide sequence to a specific cell or tissue, thereby expanding and enhancing treatment options available in numerous conditions in which more conventional therapies are of limited efficacy.

The therapeutic nucleotide sequences described herein and compositions including same have a number of uses, and may be used *in vitro* or *in vivo*. For example, the compositions may be used prophylactically or therapeutically *in vivo* to disrupt HIV infection and mechanisms of action by inhibiting gene expression or activation, via delivery of antisense HIV sequences or ribozymes to T cells or monocytes. Other useful therapeutic nucleotide sequences include antisense nucleotide sequences complementary to EBV EBNA-1 gene. Use of such therapeutic sequences may remediate or prevent latent infection of B cells with EBV. (As discussed in Example 5 below, targeting and delivery may be accomplished via the use of various ligands, receptors, and other appropriate targeting agents.)

The method comprises, in one embodiment, contacting human cells infected with EBV or HIV with a therapeutically effective amount of a pharmaceutically acceptable composition comprising a therapeutic nucleotide sequence of this invention. In a related

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embodiment, the contacting involves introducing the therapeutic nucleotide sequence composition into cells having an EBV or HIV-mediated infection.

5 The present invention also contemplates methods for determining the efficacy of the within-disclosed therapeutic compositions and methods. One such method for confirming efficacy utilizes the human/SCID (severe combined immunodeficient) mouse model of EBV-induced LPD (lymphoproliferative disease) to ascertain  
10 whether EBV-antisense therapeutic nucleotide sequences block tumor formation.

For example, it has been observed that SCID/hu chimeric mice resulting from SCID mice reconstituted with human lymphocytes from EBV positive donors  
15 develop aggressive human B-cell tumors containing viral DNA, or fatal LPD of human B-cell origin containing EBV DNA. (See, e.g., Pisa, et al., Blood 79: 173-179 (1992); Rowe, et al., Curr. Top. Microbiol. Immunol. 166: 325 (1990); and Cannon, et  
20 al., J. Clin. Invest. 85: 1333-1337 (1990)). Therefore, one means of assessing the ability of therapeutic nucleotide sequences to block EBV-associated tumor formation may be described essentially as follows. SCID mice are injected with  
25 lymphocytes from peripheral blood (PBL) or palatine tonsils (lymph nodes, LN) from EBV-seronegative individuals. The SCID/hu mice are then infected with EBV about 7 days thereafter. Administration of therapeutic nucleotide sequences may proceed prior to,  
30 concurrent with, or shortly after the EBV infection. Since EBV-infected SCID/hu mice tend to develop clinical signs of tumor 1-2 months after EBV infection, the mice are monitored over this period of time to ascertain the effect of the therapeutic  
35 nucleotide sequences and compositions on the course of



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the infection and tumor (or LPD) progression. Alternatively, the SCID/hu mice may be monitored to ascertain whether circulating cells with human and EBV DNA are present, as these cells tend to appear before  
5 the appearance of overt tumors (Pisa, et al., Blood  
79: 173-179 (1992).)

It is expressly to be understood that the above  
is an example only, as it is one of several methods  
available for determining the effectiveness of the  
10 within-described nucleotide sequences, compositions  
and therapeutic methods.

#### EXAMPLES

The following examples are intended to  
15 illustrate, but not limit, the present invention.

##### Example 1

##### Cloning, Expression and Purification of Adenovirus Type 2 Penton Base and Fiber

For the preparation of recombinant penton base  
20 and fiber from human adenovirus type 2 (hereinafter  
referred to as Ad2), PCR (polymerase chain reaction)  
amplification was performed on DNA extracted from the  
virus following propagation in HeLa cells as described  
by Horwitz, "Adenoviridae and their replication", in  
25 Virology, Fields and Knipe, eds., Raven Press, NY  
(1990) pp. 1679-1740. Briefly, HeLa cells (American  
Type Culture Collection, Rockville, MD (ATCC accession  
number CCL 2)), maintained in Eagle's MEM with  
non-essential amino acids and 90% Earle's BSS  
30 containing 10% fetal bovine serum, were infected with  
Ad2 obtained from ATCC having ATCC accession number  
VR-846 having a multiplicity of infection (MOI) of 10.  
Virus infected cells were then harvested 2-3 days  
later by pelleting by centrifugation the cells at  
35 10,000 rpm for 10 minutes at 4°C. The harvested cells

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were freeze-thawed 5 times to release intracellular particles and then the cell debris was removed by centrifugation. The resulting cell lysate was subjected to density gradient ultracentrifugation on cesium chloride (CsCl<sub>2</sub>) gradients (25-40%). The virus band was removed and dialyzed against PBS. Virus particle concentration was determined by the Bradford assay using a molecular weight of  $1.7 \times 10^8$  Daltons for adenovirus.

DNA was extracted from the propagated and purified Ad2 stocks by admixing the virus with 200 micrograms ( $\mu$ g) of proteinase K at a concentration of 10 micrograms/milliliter ( $\mu$ g/ml) and maintained at 37 degrees Celsius (37°C) for 6 hours. The admixture was then boiled for 15 minutes followed by 2 extractions with phenol/chloroform. The extracted viral DNA was then precipitated with ethanol and used as a template for PCR amplification as described below.

PCR was then performed on the Ad2 template DNA to form both amplified penton base and fiber. The 5' and 3' primers used to amplify the penton base were, respectively, 5'-TTTCTAGAAAGTATGCAGCGCGCG-3' (SEQ ID NO 2) and 5'-TTTCTAGATCAAAAAGTGCGGCT-3' (SEQ ID NO 3). The underlined nucleotides indicate the nucleotide sequence for incorporating the restriction enzyme site Xba I into the amplified penton base nucleotide sequence for subsequent cloning into the compatible Nhe I restriction site in the 14 kilobase (kb) baculovirus expression vector, pBlueBac II (Invitrogen, San Diego, CA).

The 5' and 3' primers used to amplify the fiber were, respectively, 5'-AAAGGATCCAGCTGATGAAACGCGCCA-3' (SEQ ID NO 4) and 5'-TTTGGTACCAGCTGTTATTCCTGGGCA-3' (SEQ ID NO 5). The first six underlined nucleotides

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(GGATCC) in the 5' oligonucleotide (SEQ ID NO 4) indicate the nucleotide sequence for incorporating the restriction enzyme site Bam HI into the amplified fiber nucleotide sequence. The second six underlined nucleotides (CAGCTG) with a C nucleotide overlap in the 5' oligonucleotide indicate the nucleotide sequence for incorporating the restriction enzyme site Pvu II into the amplified fiber nucleotide sequence. The 5' end of the 3' oligonucleotide (SEQ ID NO 5) contained a Kpn I (GGTACC) restriction site (the first six nucleotides). A Pvu II restriction site (CAGCTG) with a C nucleotide overlap was also incorporated into the 3' oligonucleotide as indicated by the underlined sequence. The restriction sites identified herein were amplified into the 5' and 3' ends of the fiber PCR product for cloning the resultant into the pBlueBac vector as described below.

The penton base and fiber nucleotide sequences were separately amplified by PCR in reactions containing 85 microliters ( $\mu$ l) water, 10  $\mu$ l PCR 10X buffer (100 millimolar (mM) Tris-HCl at pH 8.0, 500 mM KCl, 15 mM MgCl<sub>2</sub>, and 0.1% (w/v) gelatin), 1  $\mu$ l each of the 5' and 3' oligonucleotide primers listed above for a final concentration of 50 picomolar/ $\mu$ l, 1  $\mu$ l 100X dNTP's (final concentration of 200 micromolar ( $\mu$ M) each), 1  $\mu$ l *Thermus aquaticus* (Taq) polymerase (Perkins-Elmer Corp., Norwalk, CT), and 10<sup>9</sup> plaque forming units of purified viral DNA prepared above. Thirty amplification cycles were performed with one cycle consisting of 2 minutes at 72°C, 10 minutes at 94°C, 1 minute at 95°C, 1 minute at 60°C and 2 minutes and 15 seconds at 72°C. The PCR products were separately analyzed by gel electrophoresis to confirm that the appropriately sized DNA was amplified. For

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the penton base, the PCR product contained 1713 base pairs (bp) for encoding the penton base bordered by Xba I restriction site nucleotides. The nucleotide sequence for the penton base gene of human adenovirus type 2 has been described by Neumann et al., Gene 69:153-157 (1988). The fiber PCR product contained 1746 bp for encoding the fiber bordered by Bam HI/Pvu II and Pvu II/Kpn I restriction site nucleotides. The nucleotide sequence for the fiber gene of human adenovirus type 2 has been described by Herisse et al., Nuc. Acids Res. 9:4023-4041 (1981).

The resultant PCR products were purified and separately ligated into pBlueBac II baculovirus expression vector in the MaxBac® purchased from Invitrogen (San Diego, CA). The penton base having Xba I restriction sites on both the 5' and 3' ends was first digested with Xba I then ligated into the pBlueBac II vector previously digested with Nhe I to form a recombinant pBlueBac II penton base DNA-containing vector. Nhe I and Xba I are compatible restriction sites thereby providing for ligation of the PCR product into the expression vector. The fiber PCR product was first digested with Bam HI and Kpn I and directionally ligated into a similarly digested pBluescript vector (Stratagene, La Jolla, CA). The fiber nucleotide sequence was then removed from pBluescript with the blunt end cutter, Pvu II, after which Nhe I adapter oligonucleotides (Invitrogen) were ligated to the isolated fiber sequence. The resultant fiber nucleotide sequence having Nhe I ends was then ligated into the Nhe I digested pBlueBac II vector to form a recombinant pBlueBac II fiber DNA-containing vector.

The baculovirus expression vector is a helper-independent recombinant virus vector system

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used for expressing genes from sources including bacteria, viruses, plants and mammals. Recombinant fusion or nonfusion proteins have been reported to be expressed at levels ranging from 1-500 mg/liter.

5       The pBlueBac II expression vector was derived from the pJVNhe I expression vector described by Vialard et al., J. Virol. 64:37-50 (1990). As the pBlueBac II encodes  $\beta$ -galactosidase, the screening of recombinant baculovirus is simplified thereby not  
10       requiring cotransfection with a selectable marker-based approach. The vector has a unique Nhe I restriction cloning site and the 5' polyhedrin mRNA leader sequence of pVL941 is also utilized. The vector contains two promoters, the polyhedrin promoter  
15       of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and the p10 promoter, that are both activated very late in viral infection. The gene of interest is cloned downstream of the polyhedrin promoter which then controls synthesis of  
20       the recombinant protein. The vector also contains the lacZ gene positioned downstream of the p10 promoter which directs the synthesis of  $\beta$ -galactosidase. The two genes, the foreign gene and the lacZ gene, and their promoters, polyhedrin and p10, recombine with  
25       wild-type virus to yield recombinant virus. The recombinant virus is then plaqued; recombinant virus generates plaques that are blue and lack occlusion bodies.

30       The procedures for transfection of host cells, the subsequent screening and production of recombinant virus, and ultimately the production of recombinant protein was performed as described in the MaxBac<sup>®</sup> instruction manual provided with the commercial purchase of the pBlueBac II expression vector  
35       (Invitrogen), the disclosure of which is hereby

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incorporated by reference.

*Spodoptera frugiperda* (Sf9) cells were used for transfecting the recombinant DNA expression vectors of this invention. After 6 days post-viral infection of the cultured cells, the culture plates were examined under a dissecting microscope to select plaques that are occlusion body negative. Wild-type plaques were distinguished by the presence of occlusion bodies. Following selection of occlusion body negative recombinant plaques, the recombinant virus was purified away from contaminating wild-type virus in order to obtain a pure recombinant virus containing either penton base or fiber containing recombinants. Following purification, the selected plaques are analyzed by PCR as described above to confirm the presence of the recombined Ad2 gene and selection of the desired recombinant virus. Once the penton base and fiber recombinant virus were identified, the viruses were propagated to prepare large scale high titer virus stocks as described in the MaxBac<sup>®</sup> manual provided by Invitrogen and with procedures well known to one of ordinary skill in the art. The virus titer was then determined prior to preparation of recombinant penton base and fiber.

*Trichoplusia ni* (Bti-Tn 5B1-4) cells (hereinafter referred to as Tn 5 cells) were used to express large amounts of recombinant protein since they grow as adherent cell lines and produce recombinant proteins in amounts that surpasses that produced in Sf9 cells. Specifically, two studies identified that *Trichoplusia ni* attachment-dependent cell line, Tn 5, optimally produced 7 and 26-fold more  $\beta$ -galactosidase and secretory alkaline phosphatase per cell, respectively, than Sf9 cells. Thus, the Tn 5 cells allowed for the larger-scale protein production

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of the recombinant proteins of this invention.

The Tn 5 cells were purchased from Invitrogen and maintained in tissue culture flasks in EX-CELL 400 insect cell medium (JRH Biosciences, Lenexa, KS) supplemented with 2% fetal bovine serum (Irvine Scientific, Santa Ana, CA). The cultures were also adapted for growth in DEAE-based Dormacell 1.2 microcarrier-coated roller bottles (JRH Biosciences) for larger-scale recombinant protein production. For the latter, the microcarriers were added to PBS at 25 mg/ml and autoclaved. Fifty ml of the microcarrier suspension was added to 850 cm<sup>2</sup> polystyrene roller bottles and allowed to attach by slowly rotating the roller bottle at 0.1 rpm. After a confluent layer of microcarriers had attached to the roller bottle surface and just prior to inoculation with the cells, the roller bottle was washed 3 times with 50 ml of sterile saline solution to remove unbound microcarriers. The saline solution was then removed and a suspension of Tn 5 cells was added and allowed to attach and grow at 4 rpm. The volume of cells used for the inoculation was always kept below 100 ml to allow the cells to quickly attach to the microcarriers and to prevent cell clumping. The volume was then adjusted with fresh medium after the cells had attached which was approximately 2-3 hours later.

For expression of the recombinant proteins of this invention, Tn 5 cells were directly plated at densities of  $0.75 \times 10^4$  to  $5 \times 10^5$  cells/cm<sup>2</sup> in 24 well plates (Costar) or on wells pre-coated with Dormacell 1.2 microcarriers. At 0, 1, 2 or 3 days post-plating, the cells were separately infected with the recombinant penton base and fiber pBlueBac II vectors prepared above at a MOI of 5-30 pfu/cell. The cell

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concentration used to calculate MOI after 1, 2 and 3 days of cell growth was estimated by using the cell doubling time of 24 hours measured for Tn 5 cells in tissue culture flasks. In the other culturing conditions, Tn 5 cells were also infected with the recombinant vectors of this invention using an MOI of 5-30 pfu/cell.

To optimize the level of protein production, an initial time course was performed. T-25 flasks were seeded with Tn 5 cells at a density of  $2 \times 10^6$  cells/culture. The flasks were then infected with the high titer viral stock for both recombinant viruses at a MOI of 5. Aliquots of cells were removed over a period of 5 days and analyzed for the presence of either penton base or fiber. When the time point at which maximum protein expression was obtained, larger scale protein expression was then performed in T-150 flasks seeded with cells at a density of  $2 \times 10^6$  cells/ml and infected with the viral stock to a MOI of 5. The infected cells were then harvested by centrifugation at 4 days post-infection. The supernatant was transferred to a fresh tube and stored at 4°C. The pellet was stored at -20°C.

To purify recombinant penton base and fiber proteins from the pelleted cells, the cells were separately resuspended in 20 ml of 40 mM Tris-HCl at pH 7.65 containing the protease inhibitors (aprotinin at 1 µg/ml, PMSF at 2 mM and leupeptin at 2 µg/ml). The suspension was then vortexed and placed on ice for 10 minutes to form a cell lysate. The cell lysate was then centrifuged at 15,000 X g to pellet cell debris and form a recombinant protein-containing supernatant. Fiber protein was purified from the resultant supernatant by DEAE Sepharose (Pharmacia, Piscataway,



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NJ). The running buffer for the column chromatography was 40 mM Tris-HCl buffer at pH 7.65. The supernatants were applied to the column and the recombinant fiber protein was then eluted from the column with a linear gradient of 10 mM to 500 mM NaCl. Recombinant fiber protein eluted at 70 mM NaCl. The peak fractions were collected and pooled. The recombinant fiber protein was concentrated by Amicon filters.

The concentrated recombinant fiber protein was then further purified by chromatography over a Superose 6B column by FPLC (Pharmacia) according to manufacturer's instructions. Approximately 1-5 milligrams (mg) of recombinant fiber of greater than 90% purity was obtained following this purification procedure from 3 T-150 flasks. A single subunit of the purified individual protein was 62 kilodaltons (kD) (582 amino acids) under reducing SDS-PAGE. The fiber protein exists as a trimer of these three identical polypeptides with a resulting molecular weight of 180 kD.

Recombinant penton base was purified as described above for the fiber protein with the only exception that the penton base eluted from the DEAE Sepharose column at 170 mM NaCl following 2 washes with 80 mM NaCl. A single subunit of the purified recombinant penton base was 82 kD under reducing SDS-PAGE. Approximately 50 mg of recombinant penton base having a purity of greater than 90% was purified using the above procedure from 3 T-150 flasks. Penton base exists as a pentamer consisting of 5 identical single subunits that are noncovalently associated to form a ring-shaped complex of approximately 90 Å in diameter with a central cavity of approximately 10-20 Å.

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## Example 2

Purification of Penton Base and Fiber Proteins  
from Adenovirus Type 2A. Production of Viral Antigens

5 For purification of penton base and fiber  
proteins adenovirus type 2, a representative of  
Rosen's subgroup III is propagated in KB cell  
suspension culture. KB cells are maintained at 3 to 5  
x 10<sup>5</sup> cells in Eagle's basal medium supplemented with  
10 5% horse serum and infected with a MOI of 50 particles  
per cell. Cells are harvested 32 hours post-infection  
and washed three times in phosphate-buffered saline.  
The resultant cell pellet is resuspended in ten  
volumes hypotonic 0.01 M Tris-HCl buffer at pH 8.1, 1  
15 mM EDTA and subjected to five cycles of quick freezing  
and thawing. The cell lysate is then mixed in a  
Waring-blendor with an equal volume of fluorocarbon  
Freon 113. The aqueous phase obtained by low-speed  
centrifugation containing the viral material is  
20 centrifuged for 1 hour at 20,000 rev/min on a caesium  
chloride cushion (density, 1.43 g/ml) in a swinging  
bucket rotor (Beckman SW 27). Mature virions appear  
as a visible opalescent band at the top of the CsCl  
cushion. The band containing the virions is collected  
25 and the virus particles are further purified by  
density-gradient centrifugation in caesium chloride.  
The supernatant above the virion band in the two  
successive cycles of centrifugation, is the source of  
adenovirus-soluble antigens, namely penton base and  
30 fiber proteins.

B. Precipitation by Ammonium Sulfate

A saturated solution of ammonium sulfate at  
4°C is added to the supernatant to a final degree of  
55% saturation and the precipitate is allowed to form

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for 15 hours at 4°C and pH 6.5. The precipitate formed is centrifuged at 5000 x g for 20 minutes at 4°C and the pellet is dissolved in the minimum volume of 0.05 M sodium phosphate buffer pH 6.8 and dialyzed for 72 hours in the cold against 10 volumes of the same buffer with at least five changes. After dialysis, the viral antigen solution is cleared of possible incomplete low-density virus particles present in the centrifugation supernatant by centrifugation at 110,000 x g for 2 hours and the final supernatant or crude antigen preparation stored at -20°C until further fractionation.

C. Chromatography on Sephadex Ion Exchanger

As a second step, the crude antigen preparation dissolved in 0.05 M sodium phosphate buffer pH 6.8 is fractionated by chromatography on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals). The gel is equilibrated with 0.05 M sodium phosphate buffer pH 6.8 and poured in a jacketed column (25 x 2.5 cm) refrigerated at 4°C. The flow rate is maintained at 12 ml/hour by a peristaltic pump and the effluent collected into 3 ml fractions. Absorbance is measured at 278 nm and the gradient slope is controlled by measurement of resistivity in each fraction. A volume of crude preparation corresponding to 250 to 300 mg protein is loaded on top of the column and the gel is rinsed with 100 ml of the equilibrating buffer. This is then applied a linear sodium chloride gradient in the 0.05 M sodium phosphate buffer at pH 6.8, ranging from 0.0 to 0.5 M (500-ml total volume).

D. Chromatography on Hydroxylapatite Column

Hydroxylapatite is prepared as described by Boulanger et al., Eur. J. Biochem. 39:37-42 (1973). The hydroxylapatite gel is equilibrated by mild

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stirring in 0.01 M potassium phosphate buffer at pH 6.8 and gently poured into a column (20 x 1 cm) refrigerated at 4°C. Adenovirus-soluble antigen fractions are dialyzed against the equilibrating buffer and a volume corresponding to 25-30 mg protein is loaded on top of the hydroxyapatite column. The column is rinsed with 30 ml equilibrating buffer, then a 0.01 to 0.30 M (for penton and hexon) or 0.01 to 0.50 M (for fiber) linear gradient of potassium phosphate at pH 6.8 is applied in a total volume of 120 ml. The flow rate is 15 ml/hour and the effluent collected into 3-ml fractions. Protein concentrations are determined by the Lowry procedure with bovine serum albumin as standard.

15           E.   Polyacrylamide Gel Electrophoresis

Native samples are electrophoresed at 5 mA per tube (in disc gel) or 2.5 mA per slot (in slab gel) in 6% polyacrylamide gel (acrylamide:bisacrylamide ratio, 30:0.8) buffered with 0.375 M Tris-HCl at pH 8.9 and overlaid with a spacer gel made of 3% polyacrylamide (acrylamide:bisacrylamide ratio, 10:2.5) in 0.125 M Tris-HCl buffer at pH 6.8. The electrode buffer is 0.05 M Tris-0.384 M glycine at pH 8.3. Polypeptide analysis is carried out in sodium dodecyl sulfate (SDS)-containing polyacrylamide gel. Samples are denatured by heating for 2 minutes at 100°C in an equal volume of 4% SDS, 10% 2-mercaptoethanol, 6 M urea and analyzed on 15.5% polyacrylamide slab gel (ratio of acrylamide:bisacrylamide, 50:0.235) overlaid by a 3% spacer gel (ratio of acrylamide:bisacrylamide, 50:1.33) in the discontinuous SDS-buffer system of Laemmli (1970). Purified preparations of adenovirus type 2-derived penton base and fiber proteins thus result for use in this invention.

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**Example 3****Formation of Penton Complex with  
Purified or Recombinant Fiber and Penton Base**

For use in some aspects of this invention, a  
5 recombinant penton complex consisting of recombinant  
penton base and fiber was prepared. The purified  
recombinant penton base and fiber proteins prepared in  
Example 1 were combined in a 1:1 molar ratio in PBS at  
4°C and maintained overnight. The resultant  
10 recombinant penton complex consisting of 5 subunits of  
penton base and 3 fiber subunits per penton base were  
subsequently purified away from noncomplexed  
recombinant penton base or fiber proteins over linear  
sucrose gradients. Penton complex is also prepared by  
15 combining Ad2-purified penton base and fiber proteins  
prepared in Example 2. While the use of the  
recombinant proteins are preferred in this invention  
as they can be prepared in large quantities, the  
Ad2-purified proteins of this invention can be used in  
20 gene delivery systems instead of the recombinant  
proteins with equivalent success. Thus, although not  
specified in all of the following Examples, either the  
recombinant or the Ad2-purified proteins of this  
invention can be used to promote successful gene  
25 delivery of exogenous genes.

The confirmation of the formation of a complex  
between recombinant penton base and fiber was  
performed using by immunoprecipitation using a  
monoclonal antibody directed against the fiber  
30 protein. The procedure used was essentially as  
follows: First, purified adenovirus penton base was  
metabolically labeled with <sup>35</sup>S-methionine and purified  
on DEAE-Sepharose as described above. The labeled  
penton base was then added to unlabeled fiber to form

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a labeled complex that was immunoprecipitated by the anti-fiber monoclonal antibody Fb10. The complex was analyzed on 10% SDS gel and autoradiographed.

5 The results of this assay demonstrated that the anti-fiber antibody successfully immunoreacted and immunoprecipitated a radioactively labelled (<sup>35</sup>S-labeled) penton base bound to the fiber as compared to the following control where no significant labeled immunoprecipitable band was detected:

10 <sup>35</sup>S-labeled penton base maintained with a control protein, and then immunoprecipitated by anti-fiber monoclonal antibody Fb10; <sup>35</sup>S-labeled penton base maintained with fiber, followed by immunoprecipitation with a control antibody; and <sup>35</sup>S-labeled penton base

15 maintained with buffer only and immunoprecipitated with Fb10.

#### Example 4

##### DNA Delivery Into Mammalian Cells

20 DNA transfer has been accomplished by means of receptor-mediated endocytosis pathway as described by Wu et al., J. Biol. Chem. 262:4429-4432 (1987) and Wagner et al., Proc. Natl. Acad. Sci., USA 87:3410-3414 (1990). The use of this

25 cellular-mediated gene transfer system has certain advantages in that it is not toxic to the recipient eucaryotic cell membrane, DNA can be transferred repetitively and the DNA can be targeted to specific cells by the distribution of cell-specific receptors. For these type of transfer system, bifunctional

30 molecular conjugate gene transfer vehicles that are synthetically derived are used. One such bifunctional conjugate is transferrin and poly-L-lysine where the transferrin ligand binds to transferrin receptors on the surface of recipient cells and is then

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internalized resulting in the cotransportation of the poly-L-lysine that is a DNA-binding moiety.

This system requires the presence of specifically expressed cell surface receptors (e.g., transferrin receptors) in sufficient quantity to provide for adequate delivery of the DNA-binding moiety. However, despite the presence of an adequate number of cell surface receptors, the transfer of exogenous genes has been limited by the lysosomal targeting of the endosome-internalized ligand-DNA complexes as described by Zenke et al., Proc. Natl. Acad. Sci., USA 87:3655-3659 (1990) and Cotten et al., Proc. Natl. Acad. Sci., USA 87:4033-4037 (1990). Adenovirus has been shown to enter cells in a similar manner as that of ligand-DNA complexes but they have the capacity to disrupt the endosome thereby escaping lysosomal destruction in the cytoplasm. See, Seth et al., J. Virol. 51:650-655 (1984) and Seth et al., Mol. Cell. Biol. 4:1528-1533 (1984). More recently, replication-deficient adenovirus has been shown to augment the gene transfer of transferrin-polylysine conjugates mediated by the adenovirus-disruption of the endosome as described by Curiel et al., Proc. Natl. Acad. Sci., USA 88:8850-8854 (1991).

Earlier studies utilized recombinant adenoviruses to facilitate the transfer of genes but the system only allowed short heterologous DNA segments of less than 6 kb to be incorporated into the adenovirus genome as described by Berkner, Biotechniques 6:616-629 (1988). The system improved by Curiel et al. *supra* provided for the transfer of DNA segments of up to 15 kb without functional constraints. However, the use of replication-deficient virus to mediate transfer of foreign genes is still not optimal as co-infection of epithelial cells with wild-type

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strains of virus may result in transactivation of the replication-deficient virus which thus presents a significant safety concern for *in vivo* applications. Furthermore, whether recombinant adenoviruses are

5 capable of integrating their genome into the host cell DNA allowing for long-term stable expression of the foreign gene has not yet been determined.

The use of recombinant penton base and fiber proteins of this invention to mediate the transfer of

10 foreign genes into recipient cells both *in vitro* and *in vivo* overcomes the limitations of the above-described gene transfer systems. This invention utilizes a recombinant coat protein subunit of adenovirus, the penton, which duplicates the cell

15 receptor binding and DNA delivery properties of intact adenovirus virions and thus represents an improved method for gene therapy as well as for antisense-based antiviral therapy. The ability of penton base and fiber to mediate the transfer of exogenous or

20 non-native genes into cells was first evaluated in an *in vitro* tissue culture system. The DNA for transfer in the *in vitro* assay was a DNA plasmid containing the luciferase gene, pRSVLuc. The ligand-DNA complex, transferrin-poly-L-lysine, was also used in each

25 transfer experiment as it allowed for the detection of gene transfer from the cell endosome to the cytoplasm. Luciferase activity was then assayed at 48 hours post transfection.

For this assay, monolayers of Hela cells were

30 plated onto type IV collagen immobilized on tissue culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated fetal bovine serum, penicillin at 100 international units/ml, streptomycin at 100  $\mu$ g/ml and 2 mM

35 glutamine.



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The DNA plasmid, pRSVLuc, containing the *Photinus pyralis* luciferase gene under the control of the Rous sarcoma virus long terminal repeat enhancer/promoter was used as a reporter gene. The pRSVLuc was prepared as described by DeWet et al., Mol. Cell. Biol. 7: 725-737 (1987), the disclosure of which is hereby incorporated by reference. Other reporter genes such as those expressing  $\beta$ -galactosidase, alkaline phosphatase and chloramphenicol acetyltransferase are contemplated for use in vitro gene transfer experiments as described herein and are well known to one of ordinary skill in the art.

Human transferrin-poly-L-lysine conjugates with an average chain length of 190 lysines were synthesized as described by Wagner et al., Proc. Natl. Acad. Sci., USA 87:3410-3414 (1990) and Wagner et al., Bioconjugate Chem. 2:226-231 (1991), the disclosures of which are hereby incorporated by reference. Briefly, a solution of 102 mg (1,28  $\mu$ M) of human transferrin in 3 ml of a 30 mM sodium acetate buffer at pH 5 was subjected to gel filtration on a Sephadex G-25 column (Pharmacia). Following isolation of oxidized transferrin, the solution was added to a solution of 0.50  $\mu$ M of poly-L-lysine. The resultant formed conjugates were then purified on a Mono HR 10/10 S column (Pharmacia) with a high salt gradient elution. To prepare transferrin-poly-L-lysine-pRSVLuc complexes (also referred to as conjugate-DNA complexes), 6  $\mu$ g of pRSVLuc DNA in 350  $\mu$ l of HBS (150 mM NaCl and 20 mM Hepes at pH 7.3) were admixed with 12  $\mu$ g of the transferrin-poly-L-lysine conjugate diluted in 150  $\mu$ l of HBS. Complexes were allowed to form for 30 minutes at room temperature before admixing with cells.

For the gene transfer experiments, the cells

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prepared as described above were grown in 10 centimeter tissue culture plates until approximately 50% confluent ( $5 \times 10^6$  cells). Medium was removed and 1 ml of DMEM or Eagle's minimal essential medium containing 2% fetal bovine serum was then added. Conjugate-DNA complexes prepared above were then admixed with the cells followed by admixture of 50  $\mu$ g of either recombinant penton base, recombinant fiber or both recombinant proteins together. For experimental controls, in one case only the transferrin-poly-L-lysine without the pRSVLuc DNA or the recombinant proteins alone was added to the cells while in the other control, the conjugate-DNA complex was added in the absence of the recombinant proteins.

After the admixtures, the plates were returned to the incubator (5% CO<sub>2</sub> at 37°C) after which 3 ml of complete medium was added. After an additional 48 hours, the cells were harvested by lysis by freezing and thawing cycles for analysis of luciferase gene expression on a luminescence photometer according to manufacturer's instructions (Analytical Luminescence Laboratory, San Diego, CA).

As shown in Figure 3, DNA delivery into cells via the adenovirus penton base is illustrated. Luciferase activity was measured and plotted against sample number, as shown. The sample numbers are listed on the X-axis while luciferase activity (in light units) is plotted on the Y-axis. The samples identified by numbers 1-5 contained the following:

(1) transferrin-poly-L-lysine (shown as transferrin/PL), but no pRSVLuc; (2) pRSVLuc + transferrin/PL; (3) pRSVLuc + transferrin/PL + penton base + fiber; (4) pRSVLuc + transferrin/PL + penton base; (5) pRSVLuc + transferrin/PL + fiber.

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A DNA plasmid containing the luciferase gene, pRSVLuc was added to monolayers of HeLa cells which had been previously plated onto type IV collagen. DNA delivery was carried out in the presence of transferrin/poly L-lysine to allow detection of gene transfer from the cell endosome to the cytoplasm. Luciferase activity was assayed at 48 hours post transfection. (Refer to Example 3 for details of the gene transfer experiment.)

The samples containing recombinant penton base both demonstrated successful DNA delivery into the cells as measured by luciferase activity. Sample 4, which contained recombinant adenovirus fiber as well as penton base, displayed a somewhat greater introduction of DNA into the cells. The results of the assay using sample 5 demonstrates that adenovirus fiber alone, unaccompanied by penton base, does not appear to promote DNA delivery into cells. With HeLa cells with pRSVLuc plus transferrin-poly-L-lysine, in the absence of either penton base or fiber, no luciferase activity was detected which indicates internalization of the transferrin complex alone via transferrin receptors is not sufficient to allow gene delivery. Thus, penton base was an efficient means of mediating gene transfer in cells which do not efficiently allow gene delivery into the cytoplasm following internalization of the transferrin by the coordinate ligand.

#### Example 5

##### Construction of Penton Conjugates for Gene Delivery

Both in vitro and in vivo studies on the replication-deficient adenovirus-mediated transfer of foreign genes encoding either alpha 1-antitrypsin or cystic fibrosis transmembrane regulator protein have been performed on rat lung epithelial tissues for

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local as well as systemic use as described in NTIS Publication PB92-139260 (Pat-Appl-7-769-623), the disclosure of which is hereby incorporated by reference. The use of recombinant penton base or penton complex is contemplated within the context of the use of replication-deficient adenovirus to transfer exogenous genes into the lung for replacement of defective or nonexpressed genes. The advantages of using the recombinant proteins of this invention as described in Example 4 are applicable in this context of both *in vitro* and *in vivo* utility. For *in vitro* gene transfer, administration is accomplished by first isolating a selected cell population from a patient such as lung epithelial cells, lymphocytes and the like followed by *in vitro* gene transfer of the therapeutic compositions of this invention and the replacement of the cells into the patient. *In vivo* therapy is preferred through the administration of the therapeutic compositions of this invention by aerosol means. Also contemplated for *in vivo* applications are methods of administering therapeutic compositions of this invention by subcutaneous, intravenous, intraperitoneal, intramuscular, ocular means and the like.

One possible mode of penton-mediated gene delivery may be based on coating the penton base or penton complex with poly-L-lysine, which allows formation of a complex with DNA as well as binding to cell receptors. The low pH in the endosome causes penton base-mediated disruption of the endosomal membrane and release of the DNA plasmid into the cytoplasm.

In addition to the mode of application of penton base or penton complex in facilitating the transfer of exogenous genes into the lungs as described above,

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other means are contemplated to achieve the same results in both *in vivo* and *in vitro* applications. One gene delivery system utilizing the recombinant adenovirus penton complex results in the transfer of genes through the use of an avidin-biotin complexation reaction and an antibody bridge to either recombinant fiber, penton base or penton complex.

For this system, the required reagents include the following: either purified or recombinant-produced fiber and penton base proteins used separately or non-covalently bound into penton complex; a nonfunctional blocking antibody, preferably a monoclonal antibody, that immunoreacts with either fiber or penton base proteins so that it does not inhibit the protein's functional activity; a biotinylated CIAP (calf intestinal alkaline phosphatase expression vector) into which the exogenous or foreign gene or therapeutic DNA sequence of interest has been inserted; and a chimeric protein consisting of Protein A linked to streptavidin (referred to as SA-PA).

Since the CIAP vector DNA encodes alkaline phosphatase, the delivery of this gene into the cell allows can be measured by quantitative colorimetric assays much in the same fashion as  $\beta$ -galactosidase and the like. Biotinylation of one nucleotide on the plasmid allows its binding to the SA-PA chimeric protein in a defined way. Each SA-PA molecule can bind 4 biotin moieties linked to DNA. An expression vector capable of producing SA-PA in bacteria has been previously described by Sano et al., Science 258:120-122 (1992) and Sano et al., Bio/Technology 9:1378 (1991). Antibodies used in this invention include monoclonal and monospecific polyclonal antibodies that immunoreact with domains on the fiber

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or penton base proteins such that the protein's biologic functions are not inhibited. For example, see, Boudin et al., Viol. 92: 125-138 (1979).

5 Alternative monoclonals to nonfunctional domains of  $\alpha$ -v integrins such as LM142 described by Cheresh et al., J. Biol. Chem. 262:1434-1437 (1987) are also contemplated for used in this invention.

10 In this gene delivery system, the biotinylated DNA plasmid containing the therapeutic DNA sequence of interest binds to the streptavidin-Protein A chimeric protein. The DNA-bound SA-PA complex then binds via the PA moiety to an antibody that immunoreacts with either the fiber or penton base (also referred to as ligands). The ligands are either used separately in  
15 the noncomplexed form or used in the complexed form where fiber protein is noncovalently bound to penton base. If the fiber protein is used in the noncomplexed form to bind to an anti-fiber antibody, following the formation of the antibody and fiber  
20 bridge, the products are then admixed with penton base to form penton complex. Thus, a penton complex (also referred to as penton) is ultimately formed irrespective of whether the proteins of this invention are used in the noncomplexed form initially.

25 The formed PA-immobilized antibody-ligand complex then binds, via the functional receptor-binding site on the ligand, with the ligand-specific receptors expressed on the recipient cell surface. The resultant ligand-occupied receptors then provide for  
30 the delivery of the exogenous or foreign genes/DNA sequence(s) into the cell via the specific functions of the adenovirus-derived proteins of this invention.

In this gene delivery system described herein, the fiber protein binds to fiber-specific receptors  
35 the binding of which provides for nuclear localization

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signal targeting following receptor internalization. Following the fiber-mediated internalization process, the penton base protein, that is either admixed separately with the fiber-antibody bridge-PA-SA-DNA complex or is already noncovalently bound to fiber prior to the formation of the antibody bridge complex, provides for the enhanced uptake of DNA into the cytoplasm via the release from the endosome.

In addition to the methods of gene delivery described above, also contemplated is the use of penton base without fiber protein in the formation of the antibody-PA-SA-biotinylated DNA plasmid complex. The amino acid residue sequence present in the penton base, arginine-glycine-aspartic acid (RGD) is used to target the penton base to RGD-specific receptors expressed on cell surfaces. The RGD peptide sequence present in penton base has been shown to be the ligand binding site recognized by integrin receptors such as the vitronectin and fibronectin integrin receptors. When cells such as lymphocytes that lack RGD-specific receptors are cultured on synthetic RGD-coated plates in the presence of high concentrations of manganese, the expression of fibronectin receptor results thereby resulting in the binding of the RGD-containing penton base to the RGD-specific fibronectin receptor. The resultant receptor-penton base ligand interaction causes a consequent enhancement of internalization of occupied-cell surface receptors and increased permeability of the endosome.

Many advantages result from the use of the above-described gene/DNA delivery system. Multiple copies of the exogenous DNA in the biotinylated DNA plasmid are targeted to recipient cells. The recombinant fiber or penton base protein provides for targeting of the foreign gene to epithelial,

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endothelial, platelets and lymphoid cells expressing fiber-specific receptors. The recombinant penton base enhances the uptake of foreign DNA and the release from the endosome evading destruction by lysosomal enzymes. If the gene transfers are performed on cultured cells *in vitro* for the ultimate replacement into a patient requiring gene therapy, the cells can be plated on a synthetic RGD-coated surface that results in the upregulation of the fibronectin receptors (alpha, beta,) thereby augmenting adenovirus-protein mediated uptake. As mentioned above, targeting to nonepithelial cells, such as a patient's lymphocytes, is accomplished by plating those cells on RGD in the presence of high concentrations of manganese which upregulates fibronectin receptors that will bind penton base RGD.

Also contemplated for use in this invention are the alternative means of gene delivery where genes, DNA oligonucleotide sequences such as those encoding ribozymes, or antisense-based plasmids are conjugated directly to a region of the penton (penton base plus fiber) such that the gene delivery functions of the proteins are not inhibited. The plasmid-based vectors can either be under the control of strong constitutive promoters or regulatable promoters along with enhancer elements to obtain higher levels of gene expression than that obtained by incorporating the therapeutic gene into the intact or replication-deficient genome. Constitutive promoters include human cytomegalovirus which contains an enhancer sequence while regulatable promoters include those that are regulated by chemicals or temperature. Chemically regulatable promoters for eucaryotic expression systems include those based on induction by  $\gamma$ -interferon, heat-shock,



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heavy metal ions, and steroids such as glucocorticoids as described by Kaufman et al., In "Current Protocols in Molecular Biology", Ausubel et al., eds., Unit 16.12.5, New York (1990). For a review of strategies  
5 for expressing high-level protein expression in mammalian cells see Kaufman, Technique 2: 221-236 (1990), the disclosure of which is hereby incorporated by reference.

Alternative gene delivery systems are also  
10 contemplated where the penton is incorporated into liposomes which contain either the therapeutic foreign gene(s) or antisense oligonucleotides. An extension of this invention is that the adenovirus penton is used to target and deliver genes into nonepithelial  
15 cells by incorporating the attachment sequence (receptor-binding site) present in ligands specific for other receptors onto the fiber protein by recombinant DNA techniques for the production of a chimeric protein molecule as described herein. The  
20 chimeric molecule is referred to as a penton complex-ligand conjugate or a penton base-ligand conjugate depending on whether the proteins are used in the complexed or noncomplexed form. The chimeric molecule is then complexed via the fiber protein to  
25 the anti-fiber antibody-(PA-SA)-biotinylated DNA containing the exogenous DNA prepared as described above. The presence of preselected attachment sequences in the resultant complex provides for targeting to cells expressing specific receptors.  
30 Examples of receptors and their respective ligands or attachment sequences for use in gene delivery include the following: CR2 receptor binding to the amino acid residue attachment sequences EDPGFFNVE (SEQ ID NO 6) and EDPGKQLYNVE (SEQ ID NO 7); CD4 receptor  
35 recognizing the V3 loop of HIV gp120; transferrin

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receptor and its ligand transferrin; low density lipoprotein receptor (LDL) and its ligand; and asialoglycoproteins that recognize deglycosylated protein ligands.

5           Instead of incorporating receptor attachment sequences, antibodies specific to preselected cell surface receptors can also be used in the gene delivery system described above. Antibodies defined for use in this manner include immunoglobulin  
10           molecules and immunologically active portions of immunoglobulin molecules such as those containing an antibody combining site or paratope. Exemplary antibodies are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and  
15           portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', F(ab')<sub>2</sub> and F(v). Antibodies can be produced by conventional techniques for preparing polyclonal and monoclonal antibodies and also by recombinant DNA techniques  
20           familiar to one of ordinary skill in the art and as described in US Patent 4,816,567 and International Application WO 90/14424. Antibodies specific for cell surface receptors for use as described herein include antibodies to integrins, MHC class I and class II,  
25           asialoglycoprotein receptor, transferrin receptor, LDL receptor and the like.

          The gene delivery systems utilizing the adenovirus-derived penton base and fiber proteins of this invention are exemplary means for therapeutic  
30           intervention of common hereditary disorders involving respiratory epithelium including emphysema and cystic fibrosis. In addition, this invention is useful for the delivery of antisense compounds to epithelial cells. Since a large number of human pathogens  
35           including rhinoviruses, rotoviruses, herpesviruses,

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and papilloma virus invade the host via the epithelial cell route as a primary site of infection, the ability to target antiviral agents to epithelial cells is of therapeutic importance. The use of adenovirus-derived penton gene delivery systems is also valuable for the delivery of immunotoxin for the treatment of various cancers, including melanomas or osteosarcomas.

#### Example 6

##### Targeting Specific Cells

As discussed in the aforementioned Examples, the present invention contemplates that the therapeutic compounds and compositions of the present invention may be directed to specific receptors or cells, for the ultimate purpose of delivering those compounds and compositions to specific cells or cell types. The adenovirus-derived proteins and polypeptides of the present invention are particularly useful in this regard.

Adenovirus attachment and uptake into cells are separate but cooperative events that result from the interaction of distinct viral coat proteins with a receptor for attachment and  $\alpha_v$  integrin receptors for internalization. In the present invention, adenovirus attachment to the cell surface via the fiber coat proteins has now been discovered to be dissociable and distinct from the subsequent step of internalization, an event that depends on  $\alpha_v$  integrin binding to the RGD-containing penton base viral coat protein. These findings represent the first evidence for the existence of a virus internalization receptor which is distinct from, and functions independently of, a virus attachment receptor.

Integrins which recognize the RGD motif include  $\alpha_3\beta_1$ ,  $\alpha_{IIb}\beta_3$ , and most, if not all,  $\alpha_v$ -containing

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integrins (i.e., vitronectin receptors). Integrins have also been implicated in the attachment of certain viruses and bacteria to host cells. For example, the collagen/laminin-binding integrin,  $\alpha_2\beta_1$ , has been identified as a receptor for echovirus 1 attachment (Bergelson et al, Science 255: 1718-1720, 1992), while *Yersinia* (Isberg, Science 252: 934-938, 1991) and *Bordatella pertussis* (Relman et al, Cell 61: 1375-1382, 1990) have been shown to bind to and internalize into host cells via  $\beta_1$  and  $\beta_2$  integrins, respectively. The receptor for internalization of adenovirus has not been identified.

Vitronectin receptor is an integrin that mediates cell adhesion and other cellular receptor functions. Vitronectin receptor is a heterodimeric receptor defined by the presence of an  $\alpha_v$  subunit and a second,  $\beta$  subunit, typically  $\beta_3$  or  $\beta_5$ . Ligands for the vitronectin receptor are arg-gly-asp (RGD)-containing polypeptides or proteins, such as the adenovirus penton base.

In general, integrins may be classified in a relatively simple fashion based on their binding characteristics, as shown in Table 1.

25	Subunit	Table 1 Ligands
30	<u>Group 1</u> <sup>1</sup>	
	$\alpha_1/\beta_1$	Laminin/collagen
	$\alpha_2/\beta_1$	Collagen/laminin
35	$\alpha_3/\beta_1$	Laminin/collagen/fibronectin
	$\alpha_6/\beta_1$	Laminin
	$\alpha_6/\beta_4$	Laminin

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(Table 1, cont'd)

	<u>Group 2<sup>2</sup></u>	
5	$\alpha_4/\beta_1$	Fibronectin (CSII site)
	$\alpha_5/\beta_1$	Fibronectin (RGD site)
	$\alpha_v/\beta_1$	Fibronectin
10	$\alpha_v/\beta_3$	Fibronectin, vitronectin, fibrinogen, thrombospondin, von Willebrand's factor
15	$\alpha_v/\beta_5$	Vitronectin
	$\alpha_v/\beta_6$	Fibronectin
	$\alpha_v/\beta_8$	Unknown
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<sup>1</sup> Integrins that bind primarily to basement membrane proteins

25 <sup>2</sup> Integrins that bind primarily to matrix proteins of inflammation, wound healing, and development

30 A. Vitronectin Receptor Ligands as Targeting and Delivery Agents

Thus, as further disclosed herein, the binding interaction between penton base and the vitronectin receptor is RGD-dependent. Thus, an adenovirus penton base protein, and polypeptides derived therefrom which contain at least the RGD domain of the penton base protein, are expected to be useful in directing the therapeutic nucleotide sequences of the present invention to the vitronectin receptor.

Thus, the invention also contemplates isolated penton base protein, penton base fragments, and penton base-derived polypeptides which contain the RGD domain of adenovirus penton base. Particularly preferred are (1) isolated penton base protein described herein

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produced by recombinant DNA methods or produced by purification from adenovirus stock sources, (2) polypeptides having the RGD sequence and that substantially bind vitronectin receptor, and (3) compositions comprising one or more of the above ligands.

The present invention contemplates the use of any vitronectin receptor ligand as an active ingredient in a therapeutic composition according to the present invention, although adenovirus penton-derived proteins or polypeptides are particularly preferred. A vitronectin receptor ligand can be any polypeptide, protein, polypeptide or protein derivative, fragment or analog thereof which has the ability to substantially bind to vitronectin receptor.

"Substantially bind" in the present context means a binding affinity sufficient to be useful in the methods of the present invention. As is known in the art, useful competition for binding to a receptor depends on both the binding affinity and the concentration of ligand achievable in the vicinity of the receptor. Thus, binding affinities lower than that found on a natural ligand such as isolated adenovirus penton base are useful, so long as the cell or tissue to be treated can tolerate concentrations of ligand sufficient to compete with adenovirus binding to the receptor. Natural binding affinity of isolated penton base was determined to have a dissociation constant ( $K_d$ ) of 55 nanomolar.

A preferred adenovirus-derived protein or polypeptide has a sequence that corresponds to adenovirus strain 2 or 5 (Ad2 or Ad5). Another preferred adenovirus-derived protein or polypeptide for use as a vitronectin receptor ligand according to the present invention includes the sequence

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represented by the formula -HAIRGDTFA- (SEQ ID NO 1).

Synthesis of useful polypeptides according to the present invention can be accomplished by a variety of well known methods, including the procedures described hereinabove. Methods for identifying other suitable vitronectin receptor ligands useful in the present compositions, such as additional polypeptides based on the sequence of an adenovirus penton base amino acid residue sequence, can be identified by the use of the cell binding assays described in the above Examples.

B. Penton Base as a Targeting and Delivery Agent

1. Interaction with Integrins

The penton base coat protein contains five RGD sequences and was thus postulated to interact with cell surface integrins. To determine whether the adenovirus penton base binds to integrins, cell adhesion experiments were performed on surfaces coated with various cell matrix proteins or with recombinant penton base. The cell adhesion assay to extracellular matrix proteins and to recombinant Ad2 penton base was performed using three different cell lines. The M21 melanoma cell line was obtained from Dr. Donald Mortan (University of California at Los Angeles, CA). The M21-L4 ( $\alpha_v$ -expressing) and M21-L12 ( $\alpha_v$ -deficient) cell lines were derived from the M21-L cell line as described by Cheresh et al., J. Biol. Chem. 262: 17703-17711 (1987) and characterized for their integrin expression as previously described by Felding-Habermann et al., J. Clin. Invest. 89: 1-5 (1992). HeLa cells (ATCC Accession No CCL2) and the A549 lung carcinoma cell line (ATCC Accession No CCL185) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD).

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For the assay, the cells were first labeled with  $^3\text{H}$ -thymidine. Individual wells of 48 well non-treated cluster plates at a concentration of  $5 \times 10^4$  cells/well (Costar, Cambridge, MA) were first  
5 pre-coated overnight with varying amounts of extracellular matrix proteins or penton base. Vitronectin, laminin, fibronectin, and collagen I were purchased from Sigma (St. Louis, MO). Recombinant penton base was prepared as described in Example 1.  
10 The wells were separately coated with vitronectin ( $10 \mu\text{g/ml}$ ), collagen I ( $10 \mu\text{g/ml}$ ), laminin ( $10 \mu\text{g/ml}$ ), or penton base ( $0.5 \mu\text{g/ml}$ ).

The coated wells were then blocked with 5% BSA in PBS at pH 7.4 for 1 hour. The versene-released  
15 labeled HeLa, M21, or A549 cells resuspended in adhesion buffer (DMEM supplemented with 2 mM  $\text{MgCl}_2$ , 1% BSA, and 20 mM HEPES) were then added and allowed to attach at  $37^\circ\text{C}$  for 1 hour, the time at which adhesion reached a maximum in wells containing the most penton  
20 base and vitronectin. Unattached cells were removed by rapid washing with PBS and the amount of cell-associated radioactivity remaining in each well was determined by addition of detergent and scintillation counting the cell lysates. The  
25 percentage of attached cells was calculated from the total cell cpm added to each well.

In the cell attachment assays, HeLa, M21, and A549 cells efficiently attached to vitronectin, collagen and laminin. In addition, these cells also  
30 attached to and spread on recombinant penton base-coated wells.

To determine the effect of synthetic peptides on cell adhesion, the wells were coated with  $0.5 \mu\text{g/ml}$  penton base or  $10 \mu\text{g/ml}$  of vitronectin, laminin, or



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collagen I and then blocked with BSA. Cells were pretreated with 150 µg/ml of synthetic peptides (RGD-containing or non-RGD-containing, e.g., GRGDSP or GRGESP) in adhesion buffer for 1 hour at 4°C and then  
5 allowed to attach to the wells at 37°C for 30-60 minutes. The percentage of attached cells was determined as above.

Synthetic peptides useful as described above are commercially available. For example, RGD-containing  
10 (e.g., GRGDSP) and non-RGD-containing (e.g., GRGESP) peptides are available from Telios Pharmaceuticals, Inc. (San Diego, CA). Alternatively, synthetic peptides may be prepared via well-known techniques, such as the classical solid-phase technique described  
15 by Merrifield, Adv. Enzymol. 32: 221-296 (1969) as adapted for use with a model 430 automated peptide synthesizer (Applied Biosystems, Foster City, CA). Prepared polypeptide resins are cleaved by hydrogen  
20 fluoride, extracted and analyzed for purity by high-performance liquid chromatography (HPLC) using a reverse-phase C18 column manufactured by Waters Associates (Milford, MA).

In these competition assays, cell attachment to penton base as well as vitronectin was abrogated by  
25 incubating cells with the integrin-specific (i.e., RGD-containing) synthetic peptide, but not by a control peptide (i.e., non-RGD-containing). As expected, the RGD-containing peptide did not affect adhesion to collagen, since most collagen-mediated  
30 adhesion can be potentiated by RGD-independent integrins (Hynes, Cell 69: 11-25 (1992)). Cell attachment to all three matrix proteins and to penton base was blocked by the divalent metal ion chelator EDTA. These results suggested that penton base binds  
35 to the cell surface and this event is mediated by an

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RGD-dependent integrin receptor.

## 2. Penton Base Binds to $\alpha_v$ Integrins

To demonstrate a specific role for  $\alpha_v$  integrins in adenovirus infection, a defined pair of M21 melanoma cells were used that either express or lack  $\alpha_v$  integrins. M21-L cells specifically lack  $\alpha_v$  mRNA and protein as described by Cheresh et al., J. Biol. Chem. 262: 17703-17711 (1987). The cells were prepared as described in Section A immediately above and transfected with a full length  $\alpha_v$  cDNA, designated M21-L4 or mock-transfected, designated M21-L12. Thus, M21-L4 cells expressing  $\alpha_v$  attach to vitronectin using  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ , while M21-L12 cells, which fail to express these integrins, do not (Felding-Habermann et al., J. Clin. Invest. 89: 1-5 (1992). Both cell lines express  $\beta_1$  integrins and thus attach to collagen, laminin, and fibronectin (Felding-Habermann et al., *supra*.

In the cell attachment assay performed as described in Section A hereinabove, the adhesive properties of  $\alpha_v$ -transfected (M21-L4) and  $\alpha_v$ -negative (M21-L12) cells and inhibition by RGD-containing peptides and anti- $\alpha_v$  integrin monoclonal antibodies (also referred to as mAbs) were evaluated. Tritiated-thymidine-labeled M21-L4 and M21-L12 cells were allowed to attach to polystyrene wells precoated with concentrations ranging from 0.01 to 10  $\mu\text{g/ml}$  of vitronectin or penton base at 37°C. Binding of both cell lines to collagen I was included as a control. After 30 minutes, the wells were washed and cell adhesion was determined as the percentage of cells (cpm) attached compared with the total number of cells (cpm) added to each well. Values are the average of duplicate measurements.

Adhesion of M21-L4 or M21-L12 to polystyrene

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wells precoated with varying doses of either vitronectin or penton base was examined. M21-L4 ( $\alpha_v+$ ) but not M21-L12 ( $\alpha_v-$ ) cells adhered to vitronectin in a dose-dependent manner while both cell types bound to collagen I (data not shown). M21-L4 ( $\alpha_v+$ ) cells, but not M21-L12 ( $\alpha_v-$ ) cells, also adhered to penton base although a small amount M21-L12 binding to the highest penton base concentrations was observed. This low-level binding was inhibitable with an RGD-containing peptide (data not shown) suggesting it was due to interaction with another RGD-binding receptor, such as  $\alpha_5\beta_1$ , expressed on these cells as described by Felding-Habermann et al., *supra*.

To further substantiate the role of  $\alpha_v$  integrins in penton base binding, synthetic peptides or monoclonal antibodies (MAbs) specific for  $\alpha_v$  integrins were used to examine cell interaction with penton base or adenovirus infection of cells. M21-L4 ( $\alpha_v+$ ) cells were incubated with function-blocking MAbs to the vitronectin receptors,  $\alpha_v\beta_3$  (LM609) and  $\alpha_v\beta_5$  (P3G2), a control, non-function-blocking MAb to the  $\alpha_v$  subunit of  $\alpha_v$  integrins (LM142) or with an RGD-containing peptide and then examined for adhesion to penton base-coated surfaces. The LM142 monoclonal antibody (MAb) directed against a nonfunctional epitope of  $\alpha_v$  integrins was produced as previously described (Cheresh et al., J. Biol. Chem. 262: 1434-1437 (1987)). The MAbs LM609, P3G2, and 9227 directed to  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ , and  $\alpha$ -proteoglycan core protein respectively, were produced as previously described. See, Cheresh et al., J. Biol. Chem. 262: 17703-17711 (1987); Wayner et al., J. Cell Biol. 113: 919-929 (1991); and Bumol et al., PNAS USA 79: 1245-1249 (1982).

For the competition of cell attachment assays,

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<sup>3</sup>H-thymidine-labeled M21-L4 cells were first incubated with RGD-containing and control (i.e., non-RGD-containing) peptides (200 µg/ml) or with the indicated MABs (100 µg/ml) for 1 hour at 4°C and the cells were then added to penton base-coated wells (0.5 µg/ml) for 60 minutes at 37°C. Unattached cells were removed by washing and cell adhesion was quantitated by determining the percentage of the total cells (cpm) added that had attached (cpm). Values were the average of duplicate measurements.

The results indicated that RGD-containing peptide, but not the control peptide, inhibited M21-L4 adhesion to penton base by greater than 95% (data not shown). Cell adhesion to penton base was also significantly inhibited by a combination of the functional blocking anti- $\alpha_v$  MABs, P3G2 and LM609 but not by the non-functional MAB LM142 or by the P3G2 or LM609 antibodies alone. Similar results were obtained with HeLa and A549 cells. The MAB P4C10, directed toward the functional epitope on a  $\beta_1$  integrin, did not significantly affect adhesion of any of the cell lines to penton base. These results demonstrate that  $\alpha_v$  integrins,  $\alpha_v\beta_3$ , and/or  $\alpha_v\beta_5$ , are the primary receptors for the viral penton base coat protein.

3. Penton Base Interaction with Integrins Induces Membrane Permeabilization

a. Cell Lines and Assays

HE-p-2, HeLa and H2921 cell lines were obtained from ATCC (Rockville, MD). The CS-1 hamster melanoma cell line (Farishian, et al., Arch. Biochem. Biophys. 198: 449-461 (1979)), a generous gift of Dr. Carolyn Damsky (UCSF, San Francisco, CA) was propagated in RPMI supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 50µg/ml gentamicin. CS-1 cells do not

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adhere to vitronectin due to their failure to express  $\alpha_v$  integrins (Thomas, et al., J. Cell. Sci. 105: 191-201 (1993)). These cells express neither  $\alpha_v\beta_3$  nor  $\alpha_v\beta_5$  heterodimers on their surface yet maintain an intracellular pool of the  $\alpha_v$  integrin subunit. To generate CS-1 sublines expressing either  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  integrins on their surface, cDNAs encoding full-length  $\beta_3$  (Fitzgerald, et al., J. Biol. Chem. 262: 3936-3939 (1987)) or  $\beta_5$  (Ramaswamy, et al., EMBO J. 9: 1561-1568 (1990)) subunit proteins were subcloned into the pcDNA-1/NEO expression vector (Invitrogen, La Jolla, CA) and introduced into CS-1 cells via lipofectin-mediated transfection (Gibco-BRL, Gaithersburg, MD). Stably transformed cells were selected by growth in 500  $\mu$ g/ml Geneticin (Sigma, St. Louis, MO) and enriched from the neomycin-resistant population for the expression of either  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  by selectively propagating the adherent cell population and discarding the unattached cells during passage of the cell line.

Human adenovirus type 2 (Ad2) was propagated in HeLa or H2981 cells and was purified and stored as previously described (Everitt, et al., J. Virol. 21: 199-214 (1977)). Briefly, Ad2 was banded on cesium chloride gradients and then dialyzed against 10mM phosphate-buffered saline (PBS), pH 8.0, containing 10% glycerol and 1mM  $MgCl_2$ . Purified virus was stored at -70°C and dialyzed into the appropriate buffer just prior to use. Ad2 was labeled with [ $^3H$ ]-thymidine as previously described (Svensson, et al., J. Virol. 38: 70-81 (1981)) or with NHS-SS-biotin (Pierce, \*) as recommended by the manufacturer.

The LM142 monoclonal antibody (mAb) directed against a nonfunctional epitope of  $\alpha_v$  integrins

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(Cheresh, et al., J. Biol. Chem. 262: 1434-1437 (1987)) and the function-blocking mAbs LM609, P3G2, and P4C10 directed to  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ , and  $\beta_1$ , respectively, were produced as previously described (Cheresh, et al., J. Biol. Chem. 262: 17703-17711 (1987); Wayner, et al., J. Cell. Biol. 113: 919-929 (1991)).

Cell adhesion assays were carried out as described in sections A and B hereinabove. Ad2 virus infection was quantitated using the fluorescent focus assay (Thiel, et al., Proc. Soc. Exp. Biol. Med. 125: 892-895 (1967), incorporated by reference herein).

Binding of  $^3\text{H}$ -thymidine-labeled Ad2 or  $^{35}\text{S}$ -penton base to CS-1: $\beta_3$  and CS-1: $\beta_5$  cells was carried out as previously described (Wickham, et al., Cell 73: 303-313 (1993)). Nonspecific binding was determined in the presence of 50-fold excess unlabeled Ad2 or penton base.

Ad2 internalization into CS-1: $\beta_3$  and CS-1: $\beta_5$  cells was performed with biotinylated Ad2 using a capture ELISA as previously described by Smythe, et al. (Meth. Enzymol. 219: 223-234 (1992)). Briefly, 60  $\mu\text{g}$  of biotinylated Ad2 was added to  $1 \times 10^7$  CS-1: $\beta_3$  or CS-1: $\beta_5$  cells for 1 hour at 4°C in adhesion buffer. The unbound virus was removed by washing, and cell samples of  $1 \times 10^6$  cells each were warmed to 37°C for varying lengths of time. Uninternalized virus particles remaining on the cell surface were then "quenched" by the addition of 100  $\mu\text{g}/\text{ml}$  of soluble avidin (Boehringer-Mannheim, Indianapolis, IN) for 60 minutes at 4°C in HEPES-buffered saline containing 10mM EDTA. Internalized Ad2 was then released by solubilizing the cells with 1% NP-40 in PBS/0.2% of BSA. Cell lysates containing biotin-Ad2 were then added to ELISA wells which had been precoated with

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1µg/well of polyclonal anti-penton base IgG. Biotin-Ad2 was then detected by addition of streptavidin-alkaline phosphatase diluted 1:1000 in PBS/0.5% nonfat dry milk (Blotto) followed by chromogenic substrate.

5 The total amount of Ad2-biotin bound to the cells was determined at 4°C in the absence of soluble avidin. Nonspecific binding (less than 10% of total) was determined by analyzing cells incubated in the absence of biotin-Ad2.

10 To analyze soluble penton base internalization, radiolabeled penton base was added to cells at 4°C and then immediately warmed to 37°C. At varying times, samples were taken and diluted 10-fold in ice-cold HBSE (Hepes-buffered saline containing 10mM EDTA),  
15 washed twice in this buffer, and then resuspended in a small volume of HBSE containing 2mg/ml subtilisin (Sigma, St. Louis, MO) and incubated at 37°C for 15 minutes. Finally, the cells were washed in ice-cold adhesion buffer, and the remaining protease-resistant  
20 Ad2-associated cpm were measured. To determine the total cell associated penton base, the samples were washed three times in HBS containing 1mM CaCl<sub>2</sub> and 1mM MgCl<sub>2</sub>. Subtilisin-treated cells remained >95% viable.

25 To analyze soluble penton base internalization, radiolabeled penton base was added to cells at 4°C and then immediately warmed to 37°C. At varying times, samples were taken and diluted 10-fold in ice-cold HBSE (Hepes-buffered saline containing 10mM EDTA),  
30 washed twice in this buffer, and then resuspended in a small volume of HBSE containing 2mg/ml subtilisin (Sigma) and incubated at 37°C for 15 minutes. Finally, the cells were washed in ice-cold adhesion buffer, and the remaining protease-resistant Ad2-associated cpm were measured. To determine the total

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cell associated penton base, the samples were washed three times in HBS containing 1mM CaCl<sub>2</sub> and 1mM MgCl<sub>2</sub>. Subtilisin-treated cells remained >95% viable.

Cell permeability was assayed by <sup>3</sup>H-choline release as previously described (Seth, et al., J. Biol. Chem. 260: 9598-9602 (1985)), with minor modifications. Experiments using CS-1 cells were performed in suspension, while permeability studies with other cell lines were performed in monolayer cultures. The appropriate number of cells in adhesion buffer containing 1-5 mCi/ml <sup>3</sup>H-choline (Amersham Corp.) were incubated for 1 hour at 37°C. Cells (1 X 10<sup>5</sup> cells/sample) were then washed three times with ice-cold virus binding buffer (5mM) (Hepes buffered saline, pH 7.0, containing 0.2% BSA, 1mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, and 50mM NaN<sub>3</sub> to prevent virus internalization during warming) and incubated for 1 hour at 4°C with the appropriate virus concentration (0-50 µg/ml for dosage experiments and 0-10 µg/ml for pH and kinetic experiments). After virus binding, the cell samples were washed once with saline and then incubated for 1 hour at 37°C with 200ml permeability buffer 50mM MES (Sigma)-buffered saline, pH 6.0, containing 0.2% BSA, 1mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, and 50mM NaN<sub>3</sub>.

For pH studies, a combination of 25mM MES and 25mM HEPES was used to adjust the permeability buffer to the desired pH. After incubation in permeability buffer, the cell samples were incubated with or without adenovirus and then gently centrifuged. The percent <sup>3</sup>H-choline release was determined by measuring the counts released into the permeability buffer and the counts remaining in the cell pellet.

For experiments using adherent cells, 48-well untreated polystyrene plates were precoated with



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10mg/ml of laminin, vitronectin, or penton base in a volume of 0.2ml. Wells were then blocked with 5% BSA and then  $10^5$  HEp-2 or CS-1 cells in adhesion buffer containing 1-5 mCi/ml [ $^3$ H]-choline were added to each well. Cells became adherent within 1 hour, after which they were washed three times with virus binding buffer and then incubated with adenovirus for 1 hour at 4°C. Cells were then washed once with saline and incubated with permeability buffer for 1 hour at 37°C.  $^3$ H-choline release was determined by measuring the radioactivity in the buffer and in the adherent cells which were solubilized in 0.2ml of 1% SDS. For blocking experiments, antibodies or other soluble proteins were incubated with adherent cells for 1 hour prior to virus binding and in the subsequent virus binding and low pH incubations.

b. Results

In initial studies, the potential involvement of  $\alpha_v$  integrins in Ad2-induced membrane permeabilization was analyzed. It was reasoned that if integrins  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  were involved in virus-induced membrane permeabilization, then cells adhered to immobilized vitronectin or penton base protein should be resistant to Ad-2 mediated membrane permeabilization since their  $\alpha_v$  integrins would be redistributed to the basolateral surface and thus unavailable to the virus. Cell permeabilization studies were performed in the presence of 50mM  $\text{NaN}_3$  to prevent virus internalization, thus restricting virus interactions to the cell plasma membrane.

Human epithelial Hep-2 cells adhered to immobilized vitronectin or penton base were significantly less susceptible to Ad2-induced membrane permeabilization at pH 6.0 compared to cells adhered

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to a control matrix protein, laminin, which binds to non- $\alpha_v$  integrins (data not shown). In parallel experiments (not shown), it was found that approximately 50% of the total  $\alpha_v$  integrins were redistributed to the basolateral surface of cells adhered to vitronectin or penton base but not on cells adhered to laminin as indicated by the reduction in binding of  $^{125}\text{I}$ -labeled mAb LM142. These studies strongly suggested that penton base binding to  $\alpha_v$  integrin promotes Ad2-induced cell permeabilization.

To further examine this possibility, inhibition studies using soluble adenovirus capsid proteins or cell matrix molecules were performed (not shown). Preincubation of HEp2 cells with soluble penton base significantly inhibited Ad2-induced membrane permeabilization, while control proteins including the Ad2 hexon protein of the cell matrix protein had no effect on Ad2 membrane permeabilization.

In further studies, it was found that a number of cell lines varied in their susceptibility to Ad2-induced membrane permeabilization and that differences in susceptibility correlated with the relative levels of  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  expressed on each cell line (not shown). Therefore, to systematically investigate the role of integrins  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  in the early events in Ad2 infection, we established CS-1 cell lines expressing either  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$ , as described in Section 1 above. CS-1 cells expressing  $\alpha_v\beta_3$  (CS-1: $\beta_3$ ) or  $\alpha_v\beta_5$  (CS01: $\beta_5$ ) were established by transfecting CS-1 cells with cDNAs encoding the  $\beta_3$  or  $\beta_5$  subunit. Transfected cells were capable of adhering to immobilized vitronectin or penton base (not shown). Adhesion to penton base or vitronectin was blocked by the appropriate mAb to  $\alpha_v\beta_3$  (LM609) or  $\alpha_v\beta_5$  (P3G2) but not

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by a control antibody to  $\beta 1$  integrins (P4C10). The parental cell line, CS-1, failed to adhere to either penton base or vitronectin (data not shown). (See also Thomas, et al., J. Cell. Sci. 105: 191-201 (1993).)

The penton base binding and membrane-permeabilization-promoting properties of  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  were further examined as follows. Since M21-L4 cells express much higher levels of  $\alpha_v\beta_3$  than  $\alpha_v\beta_5$  on their cell surface, specific and saturable binding of penton base to M21-L4 cells was observed at both pH 7.4 and 5.5 ( $K_d$  = 75 vs. 55 nM; data not shown). Five-fold more penton base binding sites were detected on M21-L4 cells at pH 7.4 than pH 5.5. Penton base binding to M21-L4 cells at low pH was specific, since it could be blocked by soluble penton base and by chelation with EDTA (not shown). Since M21-L4 cells express a 20-fold higher level of  $\alpha_v\beta_3$  than  $\alpha_v\beta_5$ , these results suggested that penton base binding at low pH was mediated by  $\alpha_v\beta_3$ . Interestingly, the binding of penton base to M21-L4 at low pH was not inhibited by RGD peptides, which suggests that the RGD sequence may have a limited role in binding at low pH.

Differences in penton base binding to  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  were also observed by passing solubilized cell membrane proteins over a penton base affinity column (data not shown). Under these conditions,  $\alpha_v\beta_3$  was eluted with soluble RGD-containing peptide (e.g., GRGDSP, Telios Pharmaceuticals, Inc., San Diego, CA). In contrast,  $\alpha_v\beta_5$  could only be eluted from the penton base affinity column with EDTA. These results further indicated a difference in penton base binding to  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ . To further substantiate this finding, penton base binding to CS-1: $\beta 3$  and CS-1: $\beta 5$  cells at varying

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pH was examined (data not shown). Penton base binding to CS-1:β5 cells was relatively unaffected by changes in pH; approximately 60% of penton base binding was retained at pH 5.5-6.0. In contrast, penton base  
5 binding to CS-1:β3 cells was significantly inhibited by decreasing pH and was completely abolished at pH 5.5. These studies suggest that the structural requirements for penton base binding to α<sub>v</sub>β<sub>3</sub> differ from those for binding to α<sub>v</sub>β<sub>3</sub>. Of particular  
10 relevance, the low pH environment present in cell endosomes favors the interaction of penton base with α<sub>v</sub>β<sub>3</sub>.

C. Targeting Therapeutic Agents to Specific Cells or Receptors

15 Based on the foregoing disclosure, it is now evident that therapeutic agents (e.g., therapeutic nucleotide sequences) of the present invention may be designed to specifically target particular cell or  
20 tissue types via the alteration and refinement of particular parameters. For instance, variation of pH conditions in the delivery vehicle -- particularly if said vehicle is a liposome -- may preferentially  
25 direct the vehicle to cells expressing a particular receptor and may further facilitate entry of the vehicle and its attached or enclosed therapeutic agent into the cell. Thus, for example, administration of a  
30 therapeutic agent in conjunction with a delivery vehicle in a low pH environment may promote the interaction of the agent and vehicle (e.g., penton base + therapeutic nucleotide sequence + liposomes) with cells expressing vitronectin receptors, particularly α<sub>v</sub>β<sub>3</sub> receptors.

As discussed above, vitronectin receptor ligands such as the penton base are expected to be

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particularly useful in delivering therapeutic agents to, and into, integrin-expressing cells -- particularly, cells expressing vitronectin receptors. Further, in view of the finding that different

5 integrins, including vitronectin receptors, are expressed on different cell types and even in different locations on cells, this information is useful in designing targeting and delivery agents possessed of an elegant selectivity. (See, e.g.,

10 Hynes, Cell 69: 11-25 (1992).) For example, since adenovirus-derived proteins such as penton base or complex include one or more RGD sequences, compositions including penton base and a therapeutic agent may be preferentially targeted to integrin

15 receptor-expressing cells, such as matrix proteins involved in inflammation, wound healing, and development. More particularly, such a composition is contemplated to be useful in targeting cells expressing  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  integrins, or both.

20 Not only would such compositions be useful in the treatment of inflammatory disorders such as lupus erythematosus, rheumatoid arthritis, and the like, such compositions would be useful in targeting specific malignant, tumorigenic and transformed cells.

25 For example, as integrins have been implicated in tumor progression and metastasis (Damjanovich, et al., Am J. Respir. Cell Mol. Biol. 6: 197-206 (1992); Ruoslahti, et al., Cancer Cells 1: 119-126 (1989)), differential expression of integrins on different cell

30 lines will facilitate specific targeting. One example is provided by lung carcinoma; cell lines of the phenotype SK-LU-1 express vitronectin receptors (e.g.  $\alpha_v\beta_3$ ) (Mette, et al., Am J. Respir. Cell Mol. Biol. 8: 562-572 (1993)) and would thus be receptive to

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targeting and delivery by compositions including a vitronectin receptor ligand such as adenovirus penton base.

5 Targeting agents directed to other integrins --  
e.g. laminin, collagen, fibronectin -- are thus  
contemplated to be useful as disclosed herein, as  
well. (See, e.g., Albelda, et al., Lab. Invest. 68:  
4-17 (1993); Leavesley, et al., J. Cell Biol. 117:  
1101-1107 (1992); Cheresch, et al., Cell 57: 59-69  
10 (1989); and Felding-Haberman, et al., J. Clin. Invest.  
89: 2018-2022 (1992).)

15 The foregoing specification, including the  
specific embodiments and examples, is intended to be  
illustrative of the present invention and is not to be  
taken as limiting. Numerous other variations and  
modifications can be effected without departing from  
the true spirit and scope of the present invention.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: TARGETING AND DELIVERY OF GENES AND  
ANTIVIRAL AGENTS INTO CELLS BY THE ADENOVIRUS PENTON

(iii) NUMBER OF SEQUENCES: 7

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US 94/  
(B) FILING DATE: 03-FEB-1994

## (vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/015,225  
(B) FILING DATE: 09-FEB-1993

## (vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/046,159  
(B) FILING DATE: 13-APR-1993

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

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(x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

His Ala Ile Arg Gly Asp Thr Phe Ala  
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTCTAGAAG TATGCAGCGC GCG

23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTCTAGATC AAAAAGTGCG GCT

23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear



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(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAGGATCCA GCTGATGAAA CGCGCCA

27

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTGGTACCA GCTGTTATTC CTGGGCA

27

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Asp Pro Gly Phe Phe Asn Val Glu  
1 5

(2) INFORMATION FOR SEQ ID NO:7:

- 112 -

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 11 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu	Asp	Pro	Gly	Lys	Gln	Leu	Tyr	Asn	Val	Glu
1				5					10	

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## Claims:

1. A composition designed to specifically target epithelial cells and deliver a therapeutic nucleotide sequence to said cells, comprising an  
5 adenovirus-derived protein and said nucleotide sequence.
2. The composition of claim 1, wherein said protein is selected from the group consisting of penton base and penton complex.
- 10 3. The composition of claim 1, wherein said protein is conventionally-purified.
4. The composition of claim 1, wherein said protein is recombinant.
- 15 5. The composition of claim 1, wherein said protein is derived from adenovirus type 2.
6. The composition of claim 1, wherein said therapeutic nucleotide sequence is operatively linked to said adenovirus-derived protein.
- 20 7. The composition of claim 1, wherein said therapeutic nucleotide sequence and said adenovirus-derived protein are contained within a liposome.
8. The composition of claim 1, wherein said nucleotide sequence encodes a polypeptide and further comprises an active promoter for expressing said  
25 polypeptide.
9. The composition of claim 1, wherein said nucleotide sequence is an "antisense" sequence.
10. The composition of claim 1, further comprising a pharmaceutically acceptable carrier or  
30 excipient.
11. Use of a composition comprising an adenovirus-derived protein and a therapeutic nucleotide sequence in the manufacture of a medicament for specifically targeting epithelial cells and  
35 delivering an effective amount of said therapeutic

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nucleotide sequence to said cells.

12. The use of claim 11, wherein said therapeutic nucleotide sequence and said adenovirus-derived protein are operatively linked.

5       13. The use of claim 11, wherein said protein is selected from the group consisting of penton base and penton complex.

14. The use of claim 11, wherein said protein is conventionally purified.

10       15. The use of claim 11, wherein said protein is recombinant.

16. The use of claim 11, wherein said protein is derived from adenovirus type 2.

15       17. The use of claim 11, wherein said nucleotide sequence and said protein are incorporated into a liposome.

18. A method of making a medicament useful in specifically targeting and delivering a therapeutic nucleotide sequence to mammalian cells, comprising  
20       operatively linking an adenovirus-derived amino acid residue sequence and said nucleotide sequence and incorporating said operatively linked sequences into a liposome.

19. The method of claim 18, wherein said amino  
25       acid residue sequence comprises penton base or penton complex.

20. An article of manufacture comprising packaging material and a composition effective for targeting epithelial cells and delivering a  
30       therapeutic nucleotide sequence to said cells, wherein said composition comprises an adenovirus-derived protein and a therapeutic nucleotide sequence, and wherein said packaging material comprises a label which indicates that the composition can be used for  
35       targeting epithelial cells and delivering a

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therapeutic nucleotide sequence to said cells.

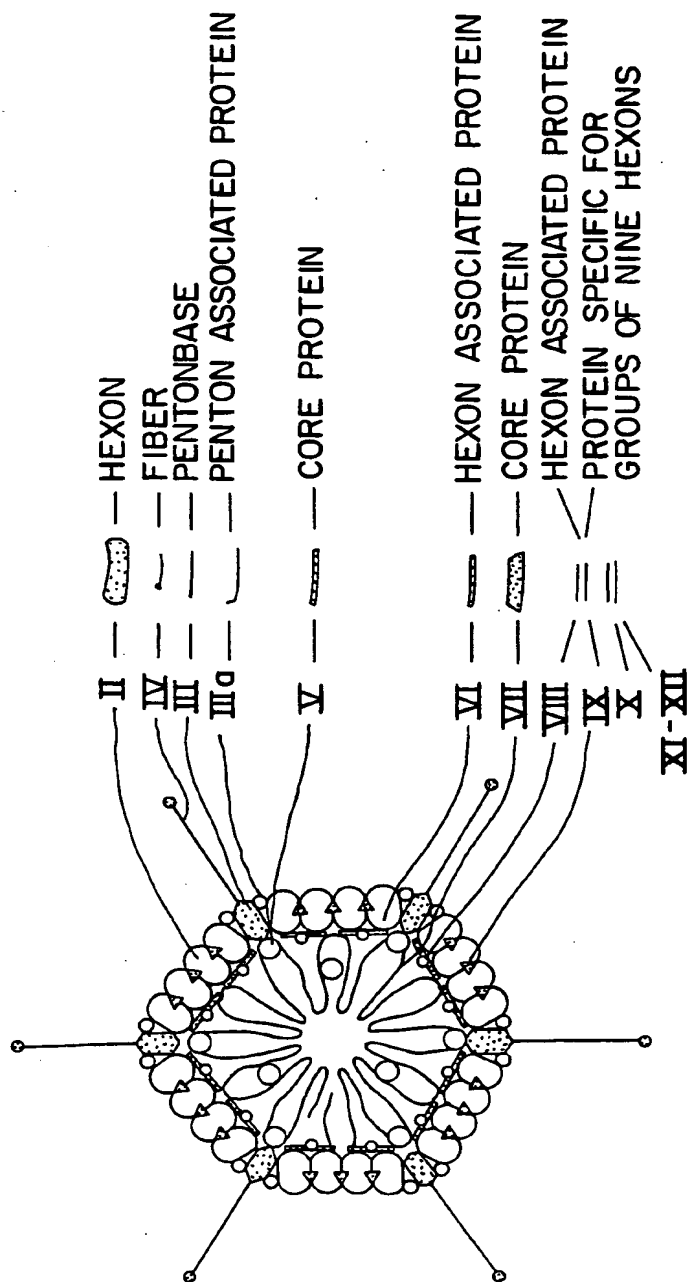
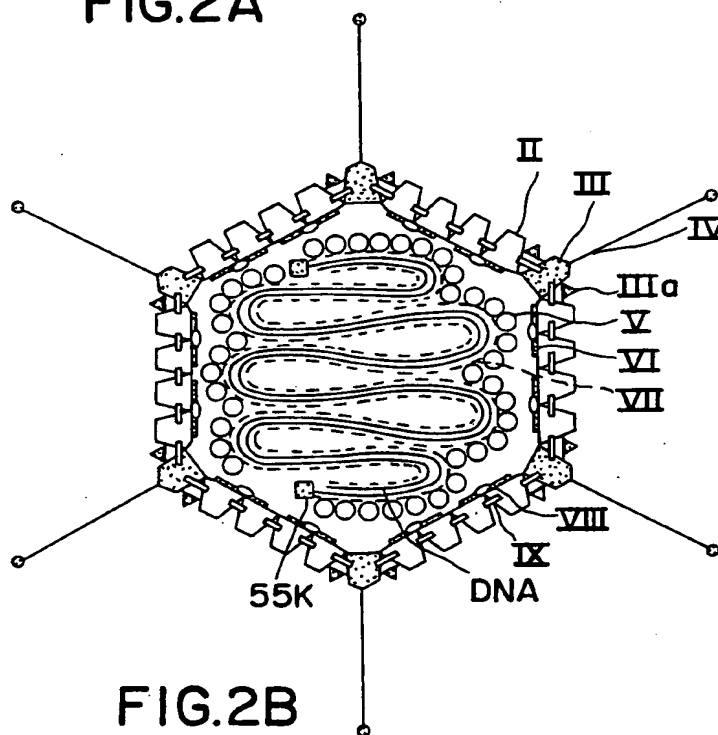
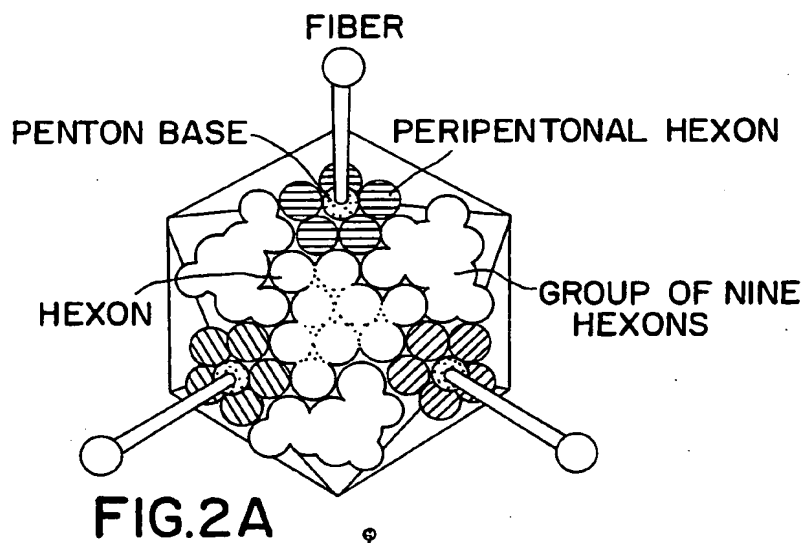


FIG. 1

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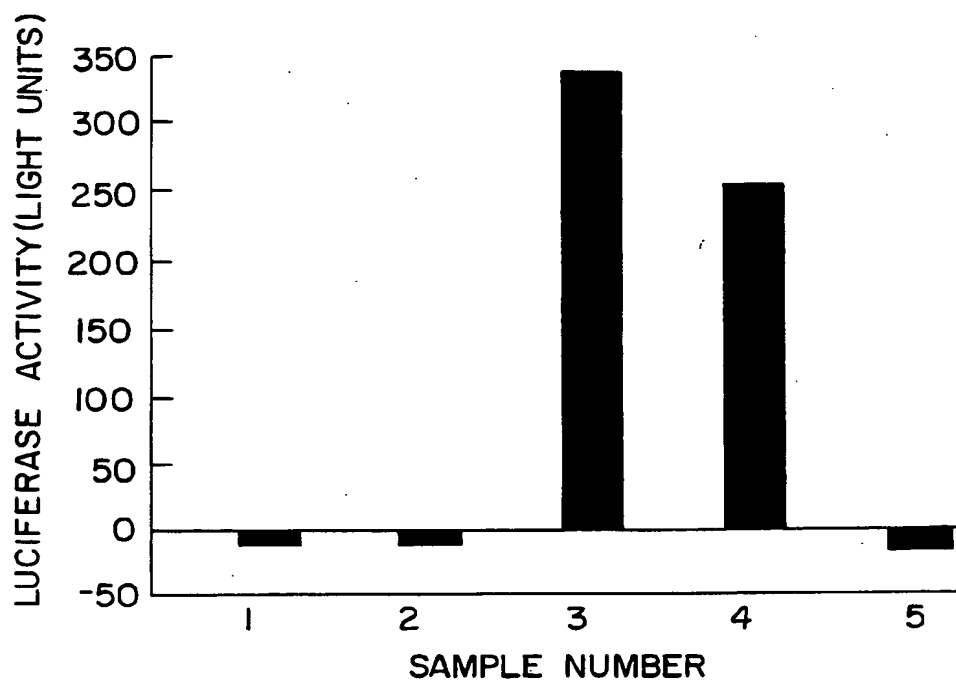
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**FIG.3**

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/01263

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 48/00; C12N 5/22, 7/04, 15/34, 15/88

US CL : 514/44, 435/69.1, 236; 935/54, 62

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44, 435/69.1, 236; 935/54, 62; 930/220

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

FUNDAMENTAL VIROLOGY

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,166,320 (Wu et al) 24 November 1992, see entire document.	1-20
Y	Journal of Virology, Volume 64, No. 8, issued August 1990, Defer et al, "Human Adenovirus-Host Cell Interactions: Comparative Study with Members of Subgroups B and C", pages 3661-3673, see entire document.	1-20
Y	PNAS USA, Vol. 88, issued October 1991, Curiel et al, "Adenovirus enhancement of transferrin-polylysine-mediated gene delivery", pages 8850-8854, see entire document.	1-20
Y	Journal of Virology, Volume 56, Number 2, issued November 1985 Jan Van Dostrum et al. "Molecular Composition of the Adenovirus Type 2 Virion"	439-448 pages the entire document.

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

08 APRIL 1994

Date of mailing of the international search report

04 MAY 1994

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